

2224/OK248

**TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)**

10/070222

INTERNATIONAL APPLICATION NO.
PCT/GB00/03306

INTERNATIONAL FILING DATE
August 25, 2000

PRIORITY DATE CLAIMED
August 27, 1999

TITLE OF INVENTION

METHOD FOR COUPLING MOLECULES

APPLICANT(S) FOR DO/EO/US

Michael John GAIT; Dmitry STETSENKO

Applicant herewith submits to the United States Designated/Elected office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S. C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371 (f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S. C. 371 (b) and PCT Articles 22 and 39 (1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S. C. 371 (c) (2))
 - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☒ has been transmitted by the International Bureau
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☐ A translation of the International Application into English (35 U.S. C. 371 (c)(2)).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☐ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c) (3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). (Unexecuted)
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☐ A FIRST preliminary amendment.
☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney an/or address letter.
16. ☐ Other items or information:

EXPRESS MAIL CERTIFICATE

0391388354

Date 2/27/02

Label No. EV

I hereby certify that, on the date indicated above, this paper or fee was deposited with the U.S. Postal Service & that it was addressed for delivery to the Assistant Commissioner for Patents, Washington, DC 20231 by "Express Mail Post Office to Addressee" service.

A. Diullo A. Diullo

Name (Print)

Signature

U.S. APPLICATION NO. (if known see 37 C.F.R. 1.50) **107 070 222** INTERNATIONAL APPLICATION NO. **307 805 038 06** Attorney's Docket Number **2224/OK248**

17. [x] The following fees are submitted:

Basic National Fee (37 CFR 1.492 (a)(1)-(5)):
 Search Report has been prepared by the EPO [X] or JPO [] **\$890.00**

International preliminary examination fee paid to USPTO (37 CFR 1.482)
 **\$710.00**

No international preliminary examination fee paid to USPTO (37 CFR 4.482)
 but international search fee paid to USPTO (37 CFR 1.445 (a) (2))... **\$740.00**

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO..... **\$1,040.00**

International preliminary examination fee paid to USPTO (37 CFR 1.482)
 and all claims satisfied provisions of PCT Article 33(2)-(4).... **\$100.00**

ENTER APPROPRIATE BASIC FEE AMOUNT =

\$890.00

Surcharge of \$130.00 for furnishing the oath or declaration later than []20 []30 months from the earliest claimed priority date (37 CFR 1.492(e)).

\$

Claims	Number Filed	Number Extra	Rate		
Total Claims	35-20		X \$18.00	\$00	
Independent Claims	7-3		X \$80.00	\$00	

Multiple dependent claims(s) (if applicable) + 280 **\$0.00**

TOTAL OF ABOVE CALCULATIONS = \$890.00

Reduction by 1/2 for filing by small entity, if applicable. **\$445.00**

SUBTOTAL = \$445.00

Processing fee of \$130.00 for furnishing the English translation later the [] 20 [] 39 months from the earliest claimed priority date (37 CFR 1.492(f)).

+

\$

TOTAL NATIONAL FEE = \$445.00

Fee for recording the enclosed assignment (37 CFR 1.21(h)). the assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **\$40.00** per property

+

\$40.00

TOTAL FEES ENCLOSED = \$445.00

Amount to be refunded \$

charged \$

a. [X] A check in the amount of **\$445.00** to cover the above fees is enclosed.

b. [] Please charge my Deposit Account in the amount of \$ to cover the above fees.

c. [] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Paul F. Fehlner, Ph.D.
 Darby & Darby P.C.
 805 Third Avenue
 New York, New York 10022-7513

SIGNATURE

Paul F. Fehlner

NAME Paul F. Fehlner, Ph.D.

REGISTRATION NO 35,135



5-124-124-1111-4
PCT

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JC04 Rec'd PCT/PTO 22 MAY 2002

Date 5/22/02 Label No. _____
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Jessica Rojas
Name (Print) Signature

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CREDIT ANY EXCESS IN THE FEES DUE WITH THIS
DOCUMENT TO OUR DEPOSIT ACCOUNT NO. 04 - 0100

Docket No: 2224/OK248

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Michael J. GAIT; Dmitry STETSENKO

Serial No: 10/070,222

Filed: February 27, 2002

For: METHOD FOR COUPLING MOLECULES

PRELIMINARY AMENDMENT

Hon. Commissioner of
Patents and Trademarks
Washington, DC 20231

Sir:

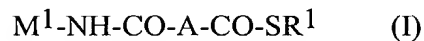
Prior to examination, please amend the above-identified application as
follows:

IN THE CLAIMS:

Please amend the claims pursuant to 37 C.F.R. §1.121 as follows:

Cancel claims 1-35 without prejudice and substitute therefor:

36. A method of linking a first molecule M^1-NH_2 with a second molecule M^2-OH , which method comprises reacting a compound of formula (I)



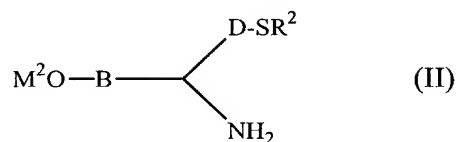
wherein

M^1 is the residue of a molecule bearing an amino group;

A is an alkylene or arylene group; and

R^1 is alkyl or aryl;

with a compound of formula (II)



wherein

M^2 is the residue of a molecule bearing a hydroxy group

B is a linker;

D is a C_{1-4} alkylene group or C_{3-12} arylene group; and

R^2 is hydrogen or a thiol protecting group.

37. A chemical compound of formula (I):



wherein

M^1 is the residue of a molecule bearing an amino group

A is an alkylene group or an arylene group; and

R^1 is selected from the group consisting of alkyl and aryl.

38. The chemical compound according to claim 37 wherein M¹ comprises a peptide residue.

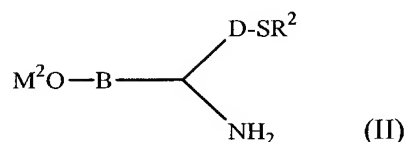
39. The chemical compound according to claim 37 wherein A comprises a C₁₋₄ alkylene group.

40. The chemical compound according to claim 38 wherein A comprises an ethylene (-CH₂-CH₂-) or n-propylene (-CH₂-CH₂-CH₂-) group.

41. The chemical compound according to claim 37 wherein R¹ comprises a C₁₋₁₈ alkyl or C₃₋₁₀ aryl group.

42. The chemical compound according to claim 41 wherein R¹ is selected from the group consisting of t-butyl, substituted or unsubstituted benzyl, substituted or unsubstituted phenyl, 2-pyridyl, 4-pyridyl, cyanomethyl carboxamidomethyl, 2-carboxamidoethyl and trifluoroethyl.

43. A chemical compound of formula (II):



wherein

M² is the residue of a molecule bearing a hydroxy group;

B is a linker;

D is a C₁₋₄ alkylene group or C₃₋₁₂ arylene group; and

R² is hydrogen or a thiol protecting group.

44. The chemical compound according to claim 43 wherein M² comprises an oligonucleotide residue.

45. The chemical compound according to claim 43 wherein the linker B comprises a group of the formula



wherein

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100 101 102 103 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120 121 122 123 124 125 126 127 128 129 130 131 132 133 134 135 136 137 138 139 140 141 142 143 144 145 146 147 148 149 150 151 152 153 154 155 156 157 158 159 160 161 162 163 164 165 166 167 168 169 170 171 172 173 174 175 176 177 178 179 180 181 182 183 184 185 186 187 188 189 190 191 192 193 194 195 196 197 198 199 200 201 202 203 204 205 206 207 208 209 210 211 212 213 214 215 216 217 218 219 220 221 222 223 224 225 226 227 228 229 230 231 232 233 234 235 236 237 238 239 240 241 242 243 244 245 246 247 248 249 250 251 252 253 254 255 256 257 258 259 260 261 262 263 264 265 266 267 268 269 270 271 272 273 274 275 276 277 278 279 280 281 282 283 284 285 286 287 288 289 290 291 292 293 294 295 296 297 298 299 300 301 302 303 304 305 306 307 308 309 310 311 312 313 314 315 316 317 318 319 320 321 322 323 324 325 326 327 328 329 330 331 332 333 334 335 336 337 338 339 340 341 342 343 344 345 346 347 348 349 350 351 352 353 354 355 356 357 358 359 360 361 362 363 364 365 366 367 368 369 370 371 372 373 374 375 376 377 378 379 380 381 382 383 384 385 386 387 388 389 390 391 392 393 394 395 396 397 398 399 400 401 402 403 404 405 406 407 408 409 410 411 412 413 414 415 416 417 418 419 420 421 422 423 424 425 426 427 428 429 430 431 432 433 434 435 436 437 438 439 440 441 442 443 444 445 446 447 448 449 450 451 452 453 454 455 456 457 458 459 460 461 462 463 464 465 466 467 468 469 470 471 472 473 474 475 476 477 478 479 480 481 482 483 484 485 486 487 488 489 490 491 492 493 494 495 496 497 498 499 500 501 502 503 504 505 506 507 508 509 510 511 512 513 514 515 516 517 518 519 520 521 522 523 524 525 526 527 528 529 530 531 532 533 534 535 536 537 538 539 540 541 542 543 544 545 546 547 548 549 550 551 552 553 554 555 556 557 558 559 560 561 562 563 564 565 566 567 568 569 570 571 572 573 574 575 576 577 578 579 580 581 582 583 584 585 586 587 588 589 590 591 592 593 594 595 596 597 598 599 600 601 602 603 604 605 606 607 608 609 610 611 612 613 614 615 616 617 618 619 620 621 622 623 624 625 626 627 628 629 630 631 632 633 634 635 636 637 638 639 640 641 642 643 644 645 646 647 648 649 650 651 652 653 654 655 656 657 658 659 660 661 662 663 664 665 666 667 668 669 670 671 672 673 674 675 676 677 678 679 680 681 682 683 684 685 686 687 688 689 690 691 692 693 694 695 696 697 698 699 700 701 702 703 704 705 706 707 708 709 710 711 712 713 714 715 716 717 718 719 720 721 722 723 724 725 726 727 728 729 730 731 732 733 734 735 736 737 738 739 740 741 742 743 744 745 746 747 748 749 750 751 752 753 754 755 756 757 758 759 760 761 762 763 764 765 766 767 768 769 770 771 772 773 774 775 776 777 778 779 780 781 782 783 784 785 786 787 788 789 790 791 792 793 794 795 796 797 798 799 800 801 802 803 804 805 806 807 808 809 810 811 812 813 814 815 816 817 818 819 820 821 822 823 824 825 826 827 828 829 830 831 832 833 834 835 836 837 838 839 840 841 842 843 844 845 846 847 848 849 850 851 852 853 854 855 856 857 858 859 860 861 862 863 864 865 866 867 868 869 870 871 872 873 874 875 876 877 878 879 880 881 882 883 884 885 886 887 888 889 890 891 892 893 894 895 896 897 898 899 900 901 902 903 904 905 906 907 908 909 910 911 912 913 914 915 916 917 918 919 920 921 922 923 924 925 926 927 928 929 930 931 932 933 934 935 936 937 938 939 940 941 942 943 944 945 946 947 948 949 950 951 952 953 954 955 956 957 958 959 960 961 962 963 964 965 966 967 968 969 970 971 972 973 974 975 976 977 978 979 980 981 982 983 984 985 986 987 988 989 990 991 992 993 994 995 996 997 998 999 1000 1001 1002 1003 1004 1005 1006 1007 1008 1009 1010 1011 1012 1013 1014 1015 1016 1017 1018 1019 1020 1021 1022 1023 1024 1025 1026 1027 1028 1029 1030 1031 1032 1033 1034 1035 1036 1037 1038 1039 1040 1

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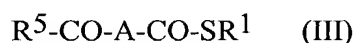
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55. The chemical compound according to claim 54 wherein the amino group is protected with a protecting group, R^4 , wherein R^4 is selected from the group consisting of urethanyl, alkyl, alkylsulfenyl, aryl sulfenyl and sulfonyl protecting groups.

56. The chemical compound of the formula (III)



wherein

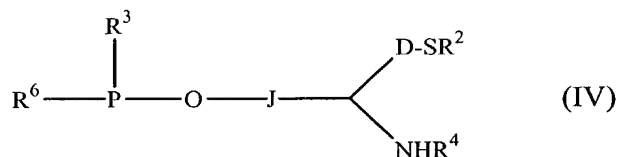
A is an alkylene group or an arylene group;

R^1 is alkyl or aryl; and

R^5 is selected from hydroxy, oxy anion and salts thereof, alkoxy, aryloxy, pentafluorophenoxy, N-succinimidyloxy, N-(norbornenedicarboximido)oxy, N-benzotriazolyloxy, N-(1,2-dihydro-1-oxo-2,3,4-benzotriazin-2-yl)oxy, halogen and N-azolyl groups; or together with the adjacent CO group forms an anhydride.

57. The chemical compound according to claim 56 wherein R^5 is a pentafluorophenoxy group.

58. The chemical compound of the formula (IV)



wherein

D is a C_{1-4} alkylene group or a C_{3-12} arylene group;

J is an alkylene group or an arylene group;

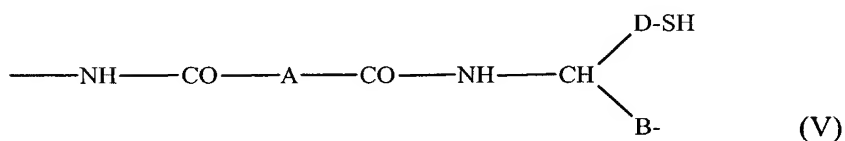
R^2 is hydrogen or a thiol protecting group;

R^3 is selected from the group consisting of hydroxy, oxy anion and salts thereof, alkyl, alkoxy, aryloxy, thiol, thioxy anion and salts thereof, S-alkyl, S-aryl, N-azolyl, and a dialkylamino group.

R^4 is selected from the group consisting of urethanyl, alkyl, alkylsulfenyl, aryl sulfenyl and sulfonyl; and

R^6 is selected from the group consisting of dialkylamino, imino, halogen N-azolyl, alkoxy, aryloxy, alkylthio, and arylthioaryl groups.

59. The chemical compound according to claim 58 wherein R^6 is a dialkylamino group.
60. The chemical compound according to claim 58 wherein R^6 is a di-isopropylamino group.
61. The chemical compound comprising a structural unit of formula (V)



wherein

A is an alkylene group or an arylene group;

B is a linker or X-J

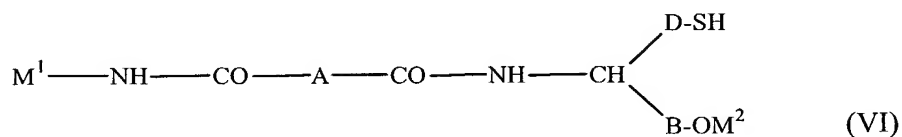
wherein

X is the residue of a functional group capable of reacting with a hydroxy group;

J is an alkylene or arylene group; and

D is a C_{1-4} alkylene group or a C_{3-12} arylene group.

62. The chemical compound according to claim 61 of the formula (VI)



wherein

M^1 is the residue of a molecule bearing an amino group or a peptide residue;

M^2 is the residue of a molecule bearing a hydroxy group;

A is an alkylene group or an arylene group;

THE UNIVERSITY OF CHICAGO

X is the residue of a functional group capable of reacting with a hydroxy group;

J is an alkylene or an arylene group; and

D is a C₁₋₄ alkylene group or a C₃₋₁₂ arylene group.

64. A method according to claim 36 wherein M¹ comprises a peptide residue.

66. A method according to claim 36 wherein B comprises a group of the formula

wherein

X is the residue of a functional group capable of reacting with a hydroxy group.

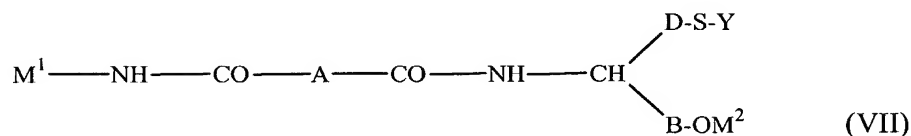
68. A method according to claim 36 wherein D is a methylene or ethylene group.

70. A method according to claim 36 wherein R¹ is selected from the group consisting of t-butyl, substituted or unsubstituted benzyl, substituted or unsubstituted phenyl, 2-pyridyl, 4-pyridyl, cyanomethyl carboxamidomethyl, 2-carboxamidoethyl and trifluoroethyl.

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72. A method according to claim 36 wherein R² is selected from the group consisting of hydrogen, alkyl, S-alkylsulfenyl, S-arylsulfenyl, alkylcarboxamidoalkyl, urethanyl and acyl groups.

73. The chemical compound of the formula (VII)



wherein

M¹ is the residue of a molecule bearing an amino group or a peptide residue;

M² is the residue of a molecule bearing a hydroxy group or an oligonucleotide residue;

A is an alkylene group or an arylene group;

B is a linker or X-J

wherein

X is the residue of a functional group capable of reacting with a hydroxy group;

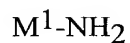
J is an alkylene or an arylene group;

D is selected from the group consisting of a C₁₋₄ alkylene group and a C₃₋₁₂ arylene group; and

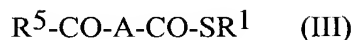
Y is a labelling, reporter or effector group.

74. A method of linking a peptide and an oligonucleotide, which method comprises reacting the peptide and the oligonucleotide with a compound of claim 37, 43, 56, 58 or 61.

75. A method of producing a compound of formula (I), comprising reacting a compound of the formula



with a chemical compound of the formula (III)



wherein

M^1 is the residue of a molecule bearing an amino group or a peptide residue;

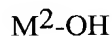
A is an alkylene group or an arylene group;

R^1 is alkyl or aryl; and

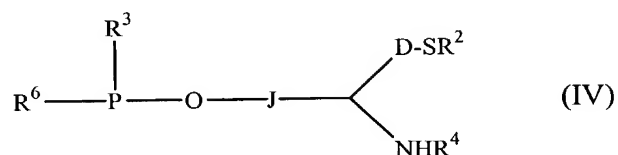
R^5 is selected from the group consisting of hydroxy, oxy anion and salts thereof, alkoxy, aryloxy, pentafluorophenoxy, N-succinimidyloxy, N-(norbornenedicarboximido)oxy, N-benzothiapoloxy, N-(1,2-dihydro-1-oxo-2,3,4-benzotriazin-z-yl) oxy, halogen or N-azolyl.

76. The method of claim 75, wherein R^5 is a pentafluorophenoxy group.

77. A method of producing a compound of formula II, comprising reacting a compound of the formula



with a chemical compound of the formula (IV)



wherein

M^2 is the residue of a molecule bearing a hydroxy group;

D is a C_{1-4} alkylene group or a C_{3-12} arylene group;

J is an alkylene group or an arylene group;

R^2 is selected from the group consisting of hydrogen and a thiol protecting group;

R^3 is a linker or X-J;

wherein

X is the residue of a functional group capable of reacting with a hydroxy group;

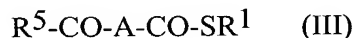
R^4 is selected from the group consisting of methanyl, alkyl, alkylsulfenyl, aryl sulfenyl and sulfonyl; and

R^6 is selected from the group consisting of dialkylamino, imino, halogen, N-azolyl, alkoxy, aryloxy, alkylthio, and arylthioaryl.

78. The method of claim 77 wherein R^6 is a dialkylamino group.

79. The method of claim 77 wherein R^6 is a di-isopropylamino group.

80. A kit comprising one or more chemical compounds wherein the compounds are selected from the compounds of formula III:



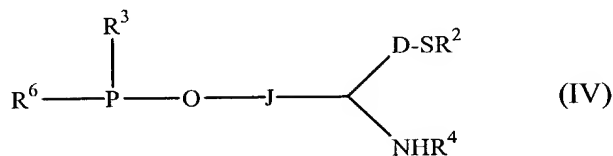
wherein

A is an alkylene group or an arylene group;

R¹ is alkyl or aryl;

R⁵ is selected from hydroxy, oxy anion and salts thereof, alkoxy, aryloxy N-succinimidyl, N-(norbornenedicarboximido)oxy, N-benzotriazolyl, N-(1,2-dihydro-1-oxo-2,3,4-benzotriazin-2-yl)oxy, halogen and N-azolyl groups; or together with the adjacent CO group forms an anhydride;

and Formula IV:



wherein

D is a C₁₋₄ alkylene group or a C₃₋₁₂ arylene group;

J is an alkylene group or an arylene group;

R² is hydrogen or a thiol protecting group;

R³ is a linker or X-J

wherein

X is the residue of a functional group capable of reacting with a hydroxy group;

R⁴ is selected from the group consisting of urethanyl, alkyl, alkylsulfenyl, aryl sulfenyl or sulfonyl; and

R⁶ is selected from dialkyl amino, imino, halogen N-azolyl, alkoxy, aryloxy, alkylthio, arylthioaryl group.

81. The kit of claim 80 wherein R⁵ is a pentafluorophenoxy group.
82. The kit of claim 80 wherein R⁶ is a dialkylamino group.
83. The kit of claim 80 wherein R⁶ is a di-isopropylamino group.

REMARKS

Claims 1-35 of the PCT application Serial No. PCT/GB00/03306 have been cancelled and rewritten as new claims 36-83 to be placed in better form for U.S. patent practice, including the removal of improper multiple dependent claims.

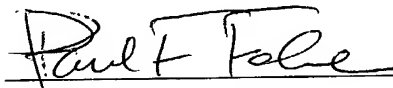
This amendment was not made for matters affecting patentability of the claims. No new subject matter has been incorporated into the application as a result of this amendment.

Entry of the above amendment is respectfully requested prior to the examination of this application.

Following entry of the amendment, claims 36-84 are presented for examination in this case. Favorable consideration and an early action on the merits is respectfully requested.

The Examiner is hereby authorized to charge any additional fees associated with this submission to our Deposit Account No. 04-0100.

Respectfully submitted,



Paul F. Fehlner, Ph.D.
Reg. No. 35,135
Agent for Applicant(s)

Dated: May 6, 2002

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New York, NY 10150-5257
Phone (212) 527-7700



1 Rec'd PGT/PTO 10 JUL 2002

EXPRESS MAIL CERTIFICATE

Date 7/10/02 Label No. EU 115469291-43

I hereby certify that, on the date indicated above, this paper or fee was deposited with the U.S. Postal Service & that it was addressed for delivery to the Assistant Commissioner for Patents, Washington, DC 20231 by "Express Mail Post Office to Addressee" service.

B.W. Lee
Name (Print)

B.W. Lee
Signature

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Customer No.:



PATENT TRADEMARK OFFICE

Docket No: 2224/OK248

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Michael J. GAIT ; Dmitry STETSENKO

Serial No.: 10/070,222

Art Unit:

Confirmation No.:

Filed: February 27, 2002

Examiner:

For: METHOD FOR COUPLING MOLECULES

PRELIMINARY AMENDMENT

Hon. Commissioner of
Patents and Trademarks
Washington, DC 20231

July 10, 2002

Sir:

In response to the Notification of Missing Requirements Under 35 U.S.C.

§ 371, dated May 10, 2002 , please amend the above-identified application as follows:

ALL INFORMATION CONTAINED HEREIN IS UNCLASSIFIED
DATE 08-16-2011 BY 60322 UCBAW

(see the accompanying "marked up" version pursuant to 1.121):

3

Table 1. Automated solid phase synthesis of peptide N-terminal S-benzyl thioesters

Sequence	MALDI-TOF MS	Purity of crude product, % (HPLC)
BnSCO-RQIPK ^{Tfa} IWFPNRRK ^{Tfa} PFK ^{Tfa} -CONH2 (SEQ ID NO: 1)	2714.59	72.0
BnSCO-GRK ^{Tfa} K ^{Tfa} RRQRRR-CONH2 (SEQ ID NO: 2)	1791.63	76.5
BnSCO-PTSQSRGDPTGPKE-CONH2 (SEQ ID NO: 3)	1660.25	88.7
BnSCO-DRVIEVVQGAYRAIRNIPRRIROG-CONH2 (SEQ ID NO: 4)	3040.62	28.6
BnSCO-QAKKKKLDK-CONH2 (SEQ ID NO: 5)	1289.92	90.3
BnSCO-ALPPLERLTL-CONH2 (SEQ ID NO: 6)	1325.70	74.7
BnSCO-GALFLGFLGAAGSTMGAWSQPKSKRKV-CO NH2 (SEQ ID NO: 7)	2969.05	48.3
BnSCO-Sar-Leu-Gly-Ile-Gly-CONH2 (SEQ ID NO: 8)	657.91	92.2
BnSCO-PQIK ^{Tfa} IWFPNRRK ^{Tfa} PFK ^{Tfa} -CONH2 (SEQ ID NO: 9)	2715.02	72.0

Delete Table 2 and insert the following Table 2.

Table 2. Automated solid phase synthesis of 5'-cysteinyl oligonucleotides

Sequence	MALDI-TOF MS	Purity of crude product, % (HPLC)
Cys(SBu ^t)-TTT TT (SEQ ID NO: 10)	1825.13	87.1
Cys(Trt)-TTT TT (SEQ ID NO: 11)	1983.16	76.6
Cys- TTT TT (SEQ ID NO: 12)	1738.24	82.2
Cys(SBu ^t)-CTC CCA GGC TCA AAT (SEQ ID NO: 13)	4874.42	82.6
Cys(SBu ^t)-GCT CCC ACG CTC AAA (SEQ ID NO: 14)	4888.15	89.7
Cys(Trt)-CTC CCA GGC TCA AAT (SEQ ID NO: 15)	5017.05	91.2
Cys(Trt)-GCT CCC AGG CTC AAA (SEQ ID NO: 16)	5043.74	89.2
Cys(Trt)-AGC TCC CAG GCT CAA (SEQ ID NO: 17)	5043.88	90.5
Cys(SBu ^t)-AGC TCC CAG GCT CAA (SEQ ID NO: 18)	4887.98	86.5
Cys-GCT CCC AGG CTC AAA (SEQ ID NO: 19)	4803.15	100

Delete Table 3 and insert the following substitute Table 3.

Table 3. Synthesis of peptide N-to-5'-oligonucleotide conjugates

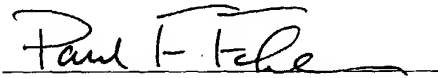
Oligonucleotide Sequence	Peptide Sequence	Method	MALDI-TO F MS	Yield %
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examination of this application.

Following entry of this amendment, favorable consideration and an early action on the merits is respectfully requested.

The Examiner is hereby authorized to charge any additional fees associated with this submission to our Deposit account No. 04-0100.

Respectfully submitted,



Paul F. Fehlner, Ph.D.

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Rec'd PCT/PTO 10 JUL 2002

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PATENT TRADEMARK OFFICE

Docket No: 2224/OK248

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Michael J. GAIT ; Dmitry STETSENKO

Serial No.: 10/070,222

Art Unit:

Confirmation No.:

Filed: February 27, 2002

Examiner:

For: METHOD FOR COUPLING MOLECULES

**MARK-UP OF SPECIFICATION FOR AMENDMENT
PURSUANT TO 37 C.F.R. §1.121**

Hon. Commissioner of
Patents and Trademarks
Washington, DC 20231

July 10, 2002

Sir:

Table 1. Automated solid phase synthesis of peptide N-terminal S-benzyl thioesters

Sequence	MALDI-TOF MS	Purity of crude product, % (HPLC)
BnSCO-RQIPK ^{Tfa} IWFPNRRK ^{Tfa} PF ^{Tfa} K ^{Tfa} -CONH2 (SEQ ID NO: 1)	2714.59	72.0
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Cys- TTT TT (SEQ ID NO: 12)	1738.24	82.2

AGC TCC CAG GCT CAA (SEQ ID NO: 25)	ALPPLRLTL (SEQ ID NO: 24)	D	6043.88	51
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METHOD FOR COUPLING MOLECULES

The present invention relates to a method for coupling molecules such as peptides and oligonucleotides, as well as synthetic intermediates and coupling agents therefor.

5

Conjugates of, for instance, peptides and oligonucleotides have many potential applications. Recently, a number of peptides have been proposed as carriers of oligonucleotides or DNA into cells. These peptides may be covalently linked to oligonucleotides via an appropriate linker.

10

The conjugation of molecules such as peptides and oligonucleotides requires reliable coupling chemistry which should be widely applicable to conjugates that vary substantially in composition. To date, there have been very few methods available for synthesising conjugates which meet these criteria. Some strategies for coupling molecules such as peptides and oligonucleotides involve synthesis and/or attaching one or both of the coupling components onto a solid support. One of the advantages of carrying out coupling reactions on solid phase is the facile purification of the product. Consequently, the technology is amenable to automated synthesis.

15

Perhaps the most ambitious route to the synthesis of peptide-oligonucleotide conjugates has been the total stepwise solid-phase approach on a single solid support. However, such a route poses serious difficulties, both technically and chemically. Firstly, no automated machine exists which can handle a sufficiently wide range of reagents to carry out the sequential assembly of peptides and oligonucleotides. Secondly, there are severe problems of compatibility in the assembly chemistries, particularly in the choice of protecting groups and deprotection conditions. Such problems have limited this approach to relatively short peptides, the longest reported conjugate produced by such a method being a 13-mer D-peptide of IGF1 coupled to a phosphorothioate 15-mer (Basu *et al*, Tetrahedron Lett., 36 (1995) 4943).

25

30

A more general strategy for peptide-oligonucleotide conjugation is where peptide and oligonucleotide moieties are assembled separately on their own solid supports and are designed to carry a reactive functionality that is released upon full deprotection and cleavage from the support. Following purification, peptide and oligonucleotide parts are
5 joined in aqueous or non-aqueous solution through the reactive functionalities. The methods currently available for coupling such functionalised peptides and oligonucleotides are somewhat limited. Amongst conjugation reactions in aqueous solution, Bongartz *et al* (Nucleic Acids Res., 22 (1994) 4681), Vives *et al* (Tetrahedron Lett., 38(1997) 1183) and Eritja *et al* (Tetrahedron Lett., 47(1991) 4113) have reported on coupling via formation of
10 a disulfide bond. Eritja *et al* (Tetrahedron, 47 (1991) 4113) have reported coupling via reaction of a thiol oligonucleotide with a maleimide peptide. Harrison *et al* (Nucleic Acids Res., 26(1998) 3136) and Tung *et al* (Bioconj. Chem., 2 (1991) 464) have reported on coupling of a cysteine peptide with a maleimido oligonucleotide. Arar *et al* (Bioconj. Chem., 6 (1995) 573) reports on the coupling of a bromoacetyl peptide with a thiol-
15 functionalised oligonucleotide. Soukchareun *et al* (Bioconj. Chem., 9 (1998) 466) have reported on coupling of a maleimido peptide with a 3'-cysteine functionalised oligonucleotide.

McMinn and Greenberg have reported the non-aqueous conjugation of a partially protected
20 peptide via its carboxyl terminus or as an aryl isocyanate derivative to a 3'-amino functionalised oligonucleotide (McMinn and Greenberg, J. Amer. Chem. Soc., 120 (1998) 3289).

Some disadvantages of performing the conjugation reaction in aqueous or non-aqueous
25 solution by published procedures are (a) the oligonucleotide often needs to be further functionalised after release from the support, (b) one or both of the coupling components often will need to be purified prior to conjugation, (c) a limitation in the peptide sequence (e.g. need for a cysteine), (d) inefficient conjugation due to secondary structure or poor solubility of the peptide or oligonucleotide components, and (e) difficulties in separation of
30 coupling components from the product conjugate, especially when conjugation yields are poor.

The solid-phase fragment conjugation method involves the retention of one component on a solid support during conjugation whilst the other remains in solution. For example Grandas *et al* have reported the fragment conjugation of a tripeptide as an amido phosphoramidite derivative to a support bound hexanucleotide (Grandas *et al*, Nucleoside and Nucleotides, 14 (1995) 825). Peyrottes *et al* have reported the conjugation of several peptides via the carboxy termini to support bound oligonucleotides which have been 5'-amino functionalised (Peyrottes *et al*, Tetrahedron, 54 (1998) 12513; Nucleosides and Nucleotides, 18 (1999) 1443). This method has several advantages over the solution-phase strategies. One advantage is that an excess of one component (e.g. peptide) can be used to drive the reaction to completion and the excess unconjugated peptide can then be removed by simple filtration and washing. This simplifies and aids the product purification following conjugation.

A highly efficient and generally applicable conjugation reaction is essential to success for both solution phase and solid-phase fragment coupling. Conjugation yields on solid phase may be greatly affected by the nature of the coupling component loaded onto the support and the nature of the coupling component in solution. Both components need to be maintained in high solvation and this needs a method of conjugation that allows the solvation conditions to be varied as appropriate (for example aqueous, aqueous-organic mixture, aqueous in the presence of denaturing agents etc).

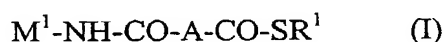
A recent method used for the synthesis of large peptides and small proteins involves "native ligation" of two largely unprotected peptide fragments (Dawson *et al*, Science, 266 (1994) 776; Wilken *et al*, Curr. Opinion Biotech., 9 (1998) 412), one containing a C-terminal thioester and the other an N-terminal cysteine.

In the case of peptide-oligonucleotide conjugations, Bruick *et al* (Chem. & Biol., 3 (1996) 49) have reported the ligation of a 3'-amino oligonucleotide to a peptide thio-ester oligonucleotide aligned on a DNA template, but the synthesis of the various components is cumbersome and the method is restricted to 3'-peptide conjugated oligonucleotides and requires a DNA template.

Consequently, there is a need to develop methods for coupling molecules such as peptides and oligonucleotides which is applicable to a wide range of substrates.

It is an object of this invention to provide a method of coupling molecules such as peptides and oligonucleotides using coupling chemistry which is both highly efficient and widely applicable. It is a further object of this invention to provide a method of coupling molecules such as peptides and oligonucleotides which can be used in solution and on solid phase and under a wide range solvent conditions that maintain solvation of the components. It is yet a further object of this invention to provide methods and reagents for modifying molecules such as peptides and oligonucleotides in order that they can be coupled together.

In accordance with the present invention there is provided a method of linking a first molecule M^1-NH_2 with a second molecule M^2-OH comprising reaction of a compound of formula (I)

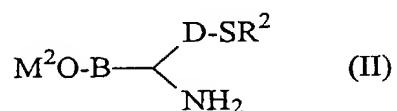


wherein M^1 is the residue of a molecule bearing an amino group,

A is an alkylene or arylene group,

R^1 is alkyl or aryl,

with a compound of formula (II)



wherein M^2 is the residue of a molecule bearing a hydroxy group

B is a linker

D is a C_{1-4} alkylene group or C_{3-12} arylene group

R^2 is hydrogen or a thiol protecting group.

The method of the present invention is of general applicability to the coupling of molecules bearing an amino group to molecules bearing a hydroxy group. Accordingly, M^1 may be the residue of any molecule bearing an amino group and M^2 may be the residue of any molecule bearing a hydroxy group. More particularly, M^1 and M^2 may each independently
5 comprise a peptide or oligonucleotide residue. Preferably, M^1 comprises a peptide residue. Preferably, M^2 comprises an oligonucleotide residue.

As used herein the term "peptide" refers to a molecule of up to 500 amino acid or peptoid (peptide-like) units. The amino acids may be naturally occurring, modified or synthetic
10 amino acids. The peptide may be fully protected, partially protected or unprotected.

As used herein the term "oligonucleotide" refers to a molecule of up to 500 nucleotide units. The nucleotide units may be naturally occurring, modified or synthetic nucleotides or peptide nucleic acids (PNAs). The oligonucleotide may contain one or more chemically
15 modified phosphate residue(s) (e.g. thiophosphate and the like) and non-phosphorus backbone substitutions (e.g. carboxamide and the like). The oligonucleotide may be fully protected, partially protected or fully unprotected.

As used herein, the term "alkyl" means a branched or unbranched, cyclic or acyclic,
20 saturated or unsaturated (e.g. alkenyl or alkynyl) hydrocarbyl radical. Where acyclic, the alkyl group is preferably a C_1 to C_{18} , more preferably C_1 to C_{10} , more preferably C_1 to C_4 chain. Where cyclic, the alkyl group is preferably a C_3 to C_{12} , more preferably C_5 to C_{10} and more preferably C_5 , C_6 or C_7 ring. The alkyl chain or ring may include (i.e. be optionally interrupted with and/or terminate with) one or more heteroatoms, such as
25 oxygen, sulfur or nitrogen.

As used herein the term "alkylene" means a branched or unbranched, cyclic or acyclic, saturated or unsaturated divalent hydrocarbyl radical. Where acyclic the alkylene group is preferably a C_1 to C_{18} , more preferably C_1 to C_{10} , more preferably C_1 to C_4 chain. Where
30 cyclic, the alkylene group is preferably a C_3 to C_{12} , more preferably C_5 to C_{10} , more

preferably C₅, C₆ or C₇ ring. The alkylene chain or ring may include (i.e. be interrupted and/or terminate with) one or more heteroatoms such as oxygen, sulfur or nitrogen.

As used herein, the term "aryl" means a C₃ to C₂₆, preferably C₃ to C₁₂, aromatic group, such as phenyl or naphthyl, or a heteroaromatic group containing one or more, preferably one, heteroatom, such as pyridyl, pyrrolyl, furanyl, thienyl.

As used herein the term "arylene" means a divalent hydrocarbyl radical comprising a C₃ to C₂₆, preferably C₃ to C₁₂, aromatic group (such as o-, m- or p-phenylene) or heteroaromatic group containing one or more, preferably one, heteroatom (such as a pyridine-2,3-diyl group).

The alkyl, aryl, alkylene and arylene groups may be further substituted or unsubstituted. For example, a C₁ (methyl) group may be further substituted with a phenyl group to give a benzyl group. Substituents may include:
carbon containing groups such as

alkyl, aryl,

aralkyl (e.g. substituted and unsubstituted phenyl, substituted and unsubstituted benzyl);

halogen atoms and halogen containing groups such as

haloalkyl (e.g. trifluoromethyl);

oxygen containing groups such as

alcohols (e.g. hydroxy, hydroxyalkyl, aryl(hydroxy)alkyl),

ethers (e.g. alkoxy, alkoxyalkyl, aryloxyalkyl),

aldehydes (e.g. carboxaldehyde),

ketones (e.g. alkylcarbonyl, alkylcarbonylalkyl, arylcarbonyl, arylalkylcarbonyl, arylcarbonylalkyl)

acids (e.g. carboxy, carboxyalkyl),

acid derivatives such as esters

(e.g. alkoxycarbonyl, alkoxycarbonylalkyl, alkylcarbonyloxy, alkylcarbonyloxyalkyl)

and amides

(e.g. aminocarbonyl, mono- or dialkylaminocarbonyl, aminocarbonylalkyl, mono- or dialkylaminocarbonylalkyl, arylaminocarbonyl);

5

and carbamates

(eg. alkoxycarbonylamino, aryloxycarbonylamino, aminocarbonyloxy, mono- or dialkylaminocarbonyloxy, arylaminocarbonyloxy),

and ureas

10

(eg. mono- or dialkylaminocarbonylamino or arylaminocarbonylamino);

nitrogen containing groups such as

amines

(e.g. amino, mono- or dialkylamino, aminoalkyl, mono- or dialkylaminoalkyl),

15

azides,

nitriles

(e.g. cyano, cyanoalkyl),

nitro;

sulfur containing groups such as

thiols, thioethers, sulfoxides, and sulfones

20

(e.g. alkylthio, alkylsulfinyl, alkylsulfonyl, alkylthioalkyl, alkylsulfinylalkyl, alkylsulfonylalkyl, arylthio, arylsulfinyl, arylsulfonyl, arylthioalkyl, arylsulfinylalkyl, arylsulfonylalkyl);

and heterocyclic groups containing one or more, preferably one, heteroatom,

25

(e.g. thienyl, furanyl, pyrrolyl, imidazolyl, pyrazolyl, thiazolyl, isothiazolyl, oxazolyl, oxadiazolyl, thiadiazolyl, pyrrolidinyl, pyrrolinyl, imidazolidinyl, imidazolinyl, pyrazolidinyl, tetrahydrofuranyl, pyranyl, pyronyl, pyridyl, pyrazinyl, pyridazinyl, piperidyl, piperazinyl, morpholinyl, thianaphthyl, benzofuranyl, isobenzofuranyl, indolyl, oxyindolyl,

30

isoindolyl, indazolyl, indolyl, 7-azaindolyl,
benzopyranyl, coumarinyl, isocoumarinyl, quinolyl,
isoquinolyl, naphthridinyl, cinnolyl, quinazolyl,
pyridopyridyl, benzoxazinyl, quinoxalinyl,
chromenyl, chromanyl, isochromanyl, phthalazinyl
and carbolinyl).

As used herein, the term "alkoxy" means alkyl-O- and "alkanoyl" means alkyl-CO.
Alkyl substituent groups or alkyl-containing substituent groups may comprise one or more
further substituents.

As used herein, the term "aryloxy" means aryl -O- and "aryloyl" means aryl -CO.
Aryl substituent groups or aryl-containing substituent groups may comprise one or more
further substituents.

As used herein, the term "halogen" means a fluorine, chlorine, bromine or iodine
radical, preferably a fluorine or chlorine radical.

According to a further aspect of the present invention there is provided a chemical
compound of formula (I). The compound of formula (I) is suitable for use in a method of
coupling M^1 -NH₂ with M^2 -OH. Preferably, M^1 comprises a peptide residue. When M^1 is a
peptide residue, the amino group in M^1 -NH₂ may be an N-terminal amino group, an
internal amino group or it may be an amino group introduced at the C-terminal end of the
peptide. Preferably, the amino group is an N-terminal amino group.

One advantage of the preparation of N-terminal thioesters is that the thioester group is
spaced away from the terminal amino acid. This is helpful in that the C-terminal thioesters
of peptides containing sterically hindered amino acids at the C-terminus, such as threonine,
isoleucine, valine or proline, are known to couple very slowly in native ligation reactions
(Hackeng *et al*, Proc. Natl. Acad. Sci. USA, (96), 1999, 10068-10073). A second

advantage is that there is no possibility of peptide racemization during conjugation reactions.

The peptide residue may be attached to a solid support. When the peptide residue is
5 attached to a solid support, the solid support may be attached to the C-terminus, internally, or the N-terminus of the peptide residue. Preferably, the solid support is attached via the C-terminus of the peptide residue.

The compounds of the present invention may be prepared, and the methods of the present
10 invention carried out, in solution phase or on a solid support, preferably on a solid support.

As used herein, the term "solid support" may be a solid support of any suitable type which will be readily apparent to those skilled in the art. Some examples of the solid supports which may be used in the present invention are polystyrene based resin such as
15 polystyrene-co-divinylbenzene resins, polyacrylamide based resin such as PEGA resin, polyethylene glycol grafted polystyrene resin such as Tentagel® resin, PEG-PS resin and NovaGel™ resin, polyethylene/polypropylene based support such as functionalised polyethylene/polypropylene pins and crowns, or silica based support such as controlled pore glass (CPG). Preferably, the solid support used for attachment to the peptide residue
20 is PAL-PEG-PS or NovaGel™ resin.

In an alternative embodiment, M¹-NH₂ comprises a peptide residue modified by introduction of an amino group at its C-terminus (by, for example, reaction of the C-terminal carboxyl group with a diaminoalkylene linker, optionally linked to a solid support,
25 generating peptide C-terminal aminoalkylamide upon deprotection (see, for example, Breipohl *et al*, Tetrahedron Lett., 28 (1987) 5647).

In a further alternative embodiment, M¹-NH₂ may comprise an oligonucleotide residue which has been modified to incorporate an amino function. For example, an amino group
30 may be introduced via an alkylene or arylene linker and phosphate or other group bonded via an oligonucleotide hydroxyl function (such as the 5' or 3' hydroxyl). Additionally, the

oligonucleotide may be linked to a solid support. Such an approach is exemplified in Nelson *et al*, Nucl. Acids Res., 17 (1989) 7179; Wachter *et al*, Nucl. Acids Res., 14 (1986) 7985; Agrawal *et al*, Nucl. Acids Res., 14 (1986) 6227. Additionally, the oligonucleotide may be linked to a solid support featuring built-in amino group liberated upon deprotection
5 (Nelson *et al*, Nucl. Acids Res., 17 (1989) 7187; Nelson *et al*, Nucl. Acids Res., 20 (1992) 6253).

In the present invention A comprises an alkylene or arylene group as defined above. Preferably A comprises a C₁₋₁₈ alkylene group, more preferably a C₂₋₄ alkylene group,
10 more preferably an ethylene or n-propylene group.

R¹ may comprise an alkyl or aryl group as defined above. Preferably, R¹ comprises a C₁₋₁₈ alkyl or C₃₋₁₀ aryl group. More preferably, the compounds of formula (I) are selected from compounds in which R¹ is t-butyl, benzyl, substituted benzyl, phenyl, substituted phenyl,
15 2-pyridyl, 4-pyridyl cyanomethyl, carboxamidomethyl, 2-carboxamidoethyl or trifluoroethyl. Most preferably R¹ is benzyl.

It will be readily apparent to the skilled person that the reactivity of the thioester can be modified by varying R¹. For instance, the compound where R¹ is phenyl will be generally
20 more reactive than the compound where R¹ is benzyl. Furthermore, the thioester of compound (I) may be transesterified *in situ* during the coupling reaction with the molecule of formula (II). The thioester may be transesterified by, for instance, thiophenol dissolved in the solvent.

25 In an alternative embodiment, R¹ further comprises a solid support. It will be appreciated that provision of a solid support permits solid phase synthesis or modification of the molecule of formula (I) by, for example, chain elongation of the peptide residue M¹ by conventional solid phase peptide synthesis techniques.

According to a further aspect of the present invention there is provided a chemical compound of formula (II). The compound of formula (II) is suitable for use in a method of coupling M^1-NH_2 with M^2-OH .

- 5 Preferably M^2 comprises an oligonucleotide residue. When M^2 is an oligonucleotide residue, the hydroxy group in M^2-OH may be a $5'$ -terminal hydroxy group, a $3'$ -terminal hydroxy group or another suitable internal hydroxy group. Preferably the oligonucleotide is bonded via its $5'$ -OH terminus.
- 10 The oligonucleotide residue may be attached to a solid support, as hereinabove defined. When the oligonucleotide residue is attached to a solid support, it may be attached via its $3'$ -OH terminus, internally or via its $5'$ -OH terminus. Preferably, when the oligonucleotide residue is part of molecule (II), it is attached via its $3'$ -OH terminus to a solid support such as controlled pore glass or polyoxyethylene-polystyrene co-polymer.

15

The group B may comprise any suitable linker. Preferably, group B comprises a group of the formula:-



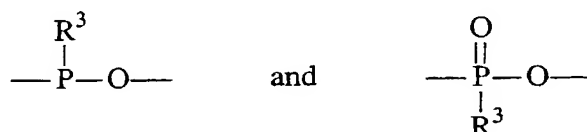
- 20 wherein J is an alkylene or arylene group and X is the residue of a functional group capable of reacting with a hydroxy group.

Preferably, J is a C_{1-18} alkylene or C_{3-12} arylene group, J may be functionalised, preferred functionalisation including carboxamido, urethane or sulfonamido groups. Preferably, J

25 comprises a moiety derived from trans-4-aminocyclohexanol or 4-hydroxypiperidine.

- X may be the residue of any suitable group capable of reacting with the hydroxyl function present M^2-OH . Preferably, X is a phosphate (including phosphoramidate), thiophosphate (including thiophosphoramidate), phosphonate or phosphite (including phosphoramidite, thiophosphite and thiophosphoramidite) residue. In case of hydrogen phosphonate and
- 30 phosphite residue, it may be optionally oxidized, e.g. by aqueous iodine solution, t-

butylhydroperoxide and the like (Letsinger *et al*, J. Am. Chem. Soc., 97 (1975) 3278), sulfurized, e.g. using molecular sulfur solution or any suitable thionating reagent (Stec *et al*, J. Am. Chem. Soc., 106 (1984) 6077), or aminated, e.g. by amine – carbon tetrachloride solution (Froehler, Tet. Lett., 27 (1986) 5575). More preferably X comprises a group
 5 selected from



wherein R³ is selected from hydroxy, oxy anion and salts thereof, alkyl alkoxy, aryloxy,
 10 thiol, thioxy anion and salts thereof, S-alkyl, S-aryl, dialkylamino and N-azolyl groups.

Preferably R³ is selected from hydroxy, oxyanion and salts thereof, alkyl (such as methyl and substituted methyl) methoxy, ethoxy and substituted ethoxy (such as 2-cyano ethoxy, 2-nitrophenylethoxy and 4-nitrophenylethoxy), allyloxy and substituted allyl, propargyloxy
 15 and substituted propargyl, benzyloxy and substituted benzyl, O-9-fluorenylmethyl, propyloxy and substituted propyloxy (such as 1,1,1,3,3,3 hexafluoroisopropyloxy), dimethylamino, diethylamino, methylethylamino, methylisopropylamino, ethylisopropylamino, diisopropylamino, di-n-propylamino, di-n-butylamino, diisobutylamino, dicyclohexylamino, pyrrolidino, piperidino, 2,6-dimethylpiperidino,
 20 2,2,6,6-tetramethylpiperidino, morpholino, 2,6-dimethylmorpholino, hexamethyleneamino, heptamethyleneamino, N-imidazolyl, substituted imidazolyl, N-benzotriazolyl, N-1,2,4-triazolyl, substituted triazolyl, N-tetrazolyl, substituted tetrazolyl, 2-chlorophenoxy, 4-chlorophenoxy, 2-nitrophenoxy, 4-nitrophenoxy, pentafluorophenoxy, 1-benzotriazolyl, thiol, thioxyanion and salts thereof, S-t-butyl, S-phenyl, S-2,4-dichlorobenzyl, or S-2,4-
 25 dinitrobenzyl.

Preferably R³ is 2-cyanoethoxy.

D may be a C₁ to C₄ alkylene group or C₃ to C₁₂ arylene group. In accordance with the definition of alkylene and arylene groups above, the groups may include one or more heteroatoms and/or heteroatomic groups such as oxygen, sulfur, nitrogen, carboxamido and the like. Preferably D is a methylene or ethylene group.

5

R² may be hydrogen or a thiol protecting group. Preferably, R² is selected from hydrogen, alkyl, S-alkylsulfenyl, S-arylsulfenyl, alkylcarboxamidoalkyl, alkoxycarbonyl and acyl groups.

- 10 Preferably R² is hydrogen, 9-fluorenylmethyl, 2-(2,4-dinitrophenyl)ethyl, t-butyl, 1-adamantyl, benzyl, substituted benzyl, benzhydryl, triarylmethyl, ethylsulfenyl, t-butylsulfenyl, tritylsulfenyl, 2-nitrobenzenesulfenyl, 2,4-dinitrobenzenesulfenyl, 3-nitro-2-pyridinesulfenyl, acetamidoethyl, trimethyl acetamidomethyl, benzamidomethyl, benzyloxycarbonyl, acetyl or benzoyl. Most preferably, R² is hydrogen, t-butylsulfenyl,
15 trityl or 4-methoxytrityl.

- When R² is other than hydrogen, the thiol group may be unmasked *in situ* during the coupling reaction with the molecule of formula (I). The *in situ* deprotection conditions will be readily apparent to the skilled person. For instance, when R² is t-butylsulfenyl, the thiol
20 group can be unmasked by reductive cleavage of the disulfide bond using tris-(2-carboxyethyl)phosphine (TCEP) dithiothreitol (DTT) or other suitable reducing agent.

- In a further aspect of the present invention there is provided a compound of formula II in which the amino group is protected. Any suitable protecting group R⁴ may be used.
25 Preferably, the protecting group R⁴ is selected from urethanyl, alkyl, alkylsulfenyl, arylsulfenyl and sulphonyl protection groups.

- Preferably R⁴ is 9-fluorenylmethoxycarbonyl, allyloxycarbonyl, propargyloxycarbonyl, t-butyloxycarbonyl, benzyloxycarbonyl, 2-(2-nitrophenyl)ethoxycarbonyl, 2-(4-nitrophenyl)ethoxycarbonyl,
30 2-(2,4-dinitrophenyl)ethoxycarbonyl, heteroarylmethoxycarbonyl, diarylmethyl, triarylmethyl, trityl, 2,6-dioxocyclohexyliden-1-

ylmethyl, substituted 2,6-dioxocyclohexyliden-1-ylmethyl, 2-nitrobenzenesulfonyl, 2,4-dinitrobenzenesulfonyl, 3-nitro-2-pyridinesulfonyl, substituted arenesulfonyl, 2-nitrobenzenesulfonyl, 2,4-dinitrobenzenesulfonyl. Most preferably, R⁴ is 9-fluorenylmethoxycarbonyl.

5

When R⁴ is other than hydrogen, the amino group may be unmasked *in situ* during the coupling reaction with the molecule of formula (I). The *in situ* deprotection conditions will be readily apparent to the skilled person. For instance, when R⁴ is, 9-fluorenylmethoxycarbonyl (Fmoc), the amino group can be unmasked under mildly basic
10 conditions such as 20% (v/v) pyridine in dimethylformamide.

Either of the compound of formula (I) or (II), but not both, may be attached to a solid support during the coupling reaction. The resultant conjugate may be cleaved from the solid support by methods known in the art.

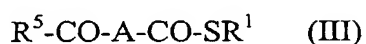
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Alternatively, the coupling of molecules (I) and (II) may be performed in solution. The solution may be either aqueous or non-aqueous, or may comprise a mixture of aqueous and non-aqueous solvents with or without denaturing agent such as urea or guanidinium chloride. The solution may optionally be buffered with a suitable buffer. The solution may
20 optionally comprise, reagent(s) for the transesterification of the thioester in molecule (I), e.g. thiophenol, and/or reagent(s) for the deprotection of the thiol group in molecule (II), e.g. TCEP.

The coupling of molecules (I) and (II) in solution may be optimised by an appropriate
25 choice of solvent, concentration of molecules (I) and (II), and pH. For example, it has been found that the use of acetonitrile or DMF as co-solvent, a high concentration (greater than 0.1 mM, but a lower concentration of 0.001 to 0.1 mM can also be used) of oligonucleotide, and a solution buffered to about pH 6.5 are preferred conditions for coupling oligonucleotides with peptides which are susceptible to a cyclisation side-
30 reaction. A possible undesired cyclisation side-reaction involves attack of a deprotonated N-terminal amido group of the peptide on the thioester carbonyl group. Such a side-

reaction cannot take place in the case of, for example, N-terminal proline or N-terminal N-methyl glycine (sarcosine), since the amido group in each case is secondary and does not contain an acidic hydrogen atom.

- 5 According to a further aspect of the present invention there is provided a chemical compound of formula (III) and its use in a method of producing a compound of formula (I) by reaction with M^1-NH_2 .



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wherein A and R^1 are as previously defined, and

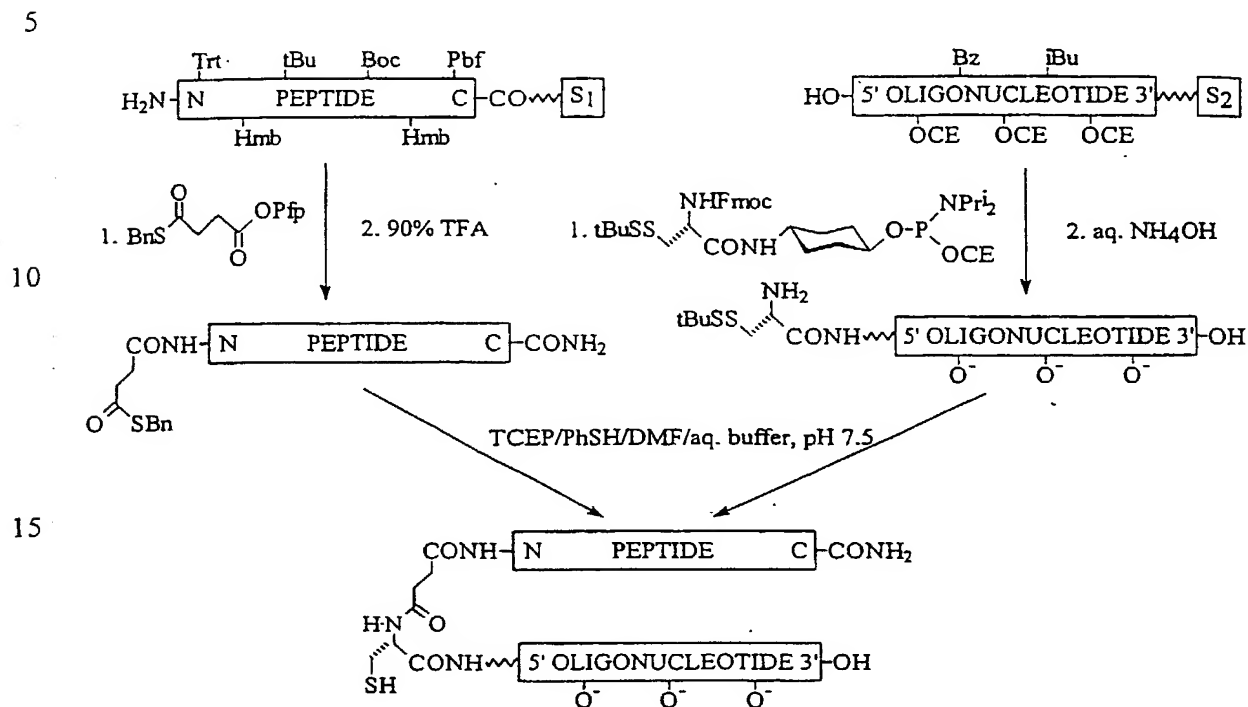
R^5 is selected from hydroxy, oxy anion and salts thereof, alkoxy, aryloxy N-succinimidyl, N-(norbornenedicarboximido)oxy, N-benzotriazolyl, N-(1,2-dihydro-1-oxo-2,3,4-benzotriazin-2-yl)oxy, halogen and N-azolyl groups; or
15 together with the adjacent CO group forms an anhydride.

Preferably, R^5 is selected from the group consisting of hydroxy, oxy anion and salts thereof, alkoxy (such as 2-cyanoethoxy), substituted or unsubstituted aryloxy (such as 2-nitrophenoxy, 4-nitrophenoxy, 2,4,5-trichlorophenoxy, pentachlorophenoxy, 2,3,5,6-
20 tetrafluorophenoxy and pentafluorophenoxy), substituted or unsubstituted N-(succinimidyl)oxy (such as N-(2-sulfosuccinimidyl)oxy (which is particularly preferred as it improves solubility and maintains reactivity in aqueous and aqueous-organic solutions) and N-(norbornenedicarboximido)oxy, N-(benzotriazolyl)oxy, substituted N-(benzotriazolyl)oxy (such as N-(7-azabenzotriazdyl)oxy, N-(1,2-dihydro-1-oxo-2,3,4-
25 benzotriazin-2-yl)oxy, substituted N-(1,2-dihydro-1-oxo-2,3,4-benzotriazin-2-yl)oxy, halogen (such as fluorine and chlorine), N-azolyl groups (such as N-imidazolyl and substituted imidazolyl, N-benzotriazolyl, N-1,2,4-triazolyl, substituted triazolyl, N-tetrazolyl, substituted tetrazolyl and azido); or together with the adjacent CO group forms a symmetrical anhydride, or a mixed anhydride with carboxylic acids (such as
30 trimethylacetic, 1-adamantane carboxylic or isovaleric), carbonic acid phosphoric or thiophosphoric acids (such as dimethylphosphoric, diethylphosphoric or

diphenylphosphoric acids), phosphonic acids (such as n-propylphosphonic acid), phosphinic or thiophosphinic acids (such as dimethylphosphinic, dimethyl thiophosphinic, diphenylphosphinic or diphenylthiophosphinic acids), sulfonic acids (such as benzene and substituted benzene sulfonic acids, methane sulfonic acid, trifluoromethane-sulfonic acid, 5 trifluoroethane sulfonic acid and polymeric poly (fluorocarbon) sulfonic acid) or hydrocyanic acid.

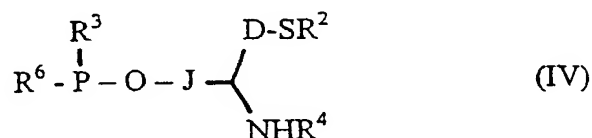
The reaction between a coupling reagent of formula (III) and the amino group of a peptide residue or an amino-modified oligonucleotide residue will chemoselectively form an amide 10 bond between the amino group and the coupling reagent (III) by overall displacement of R^5 . Accordingly, R^5 should be chosen such that the adjacent carbonyl is more reactive to amino groups than the carbonyl of the thioester. The choice of R^1 and R^5 to achieve the desired chemoselectivity will be readily apparent the skilled person. The choice of R^5 is also influenced by the intended medium for the reaction with M^1-NH_2 . For example when 15 M^1 is a peptide residue selection of R^5 as an N-succinimidyloxy group (such as N-(2-sulfosuccinimidyl)oxy) is particularly preferred as it improves solubility in aqueous-organic mixtures. In non-aqueous media, R^5 is preferably pentafluorophenoxy while R^1 is benzyl.

The preparation and coupling of molecules of general formulae (I) and (II) is exemplified in Reaction Scheme 1. It will be appreciated that preparation and coupling of molecules of general formula (I) and (II) may be carried out by modification of the procedures shown in Reaction Scheme 1 in accordance with conventional synthetic organic chemistry.



Reaction Scheme 1

According to a further aspect of the present invention there is provided a chemical compound of formula (IV) and its use in producing a compound of formula (II) by reaction with M^2 -OH.



wherein D, J, R^2 , R^3 and R^4 are as previously defined; and R^6 is selected from dialkylamino (such as dimethylamino, diethylamino, methylethylamino, methylisopropylamino, ethylisopropylamino, diisopropylamino di-n-propylamino, di-n-

30

butylamino, diisobutylamino, dicydonexylamino, pyrrolideno, piperidino, 2,6-demethylpiperidino, 2,2,6,6-tetramethyl-piperidino, morpholino and 2,6-dimethylmorpholino); imino (such as hexamethylene imino and heptamethylene imino); halogen (such as fluorine and chlorine); N-azolyl (such as N-imidazolyl and substituted imidazolyl, N-benzotriazolyl, N-1,2,4-triazolyl and substituted triazolyl, N-tetrazolyl and substituted N-tetrazolyl); alkoxy (such as 1,1,1,3,3,3-hexafluoroisopropoxy); aryloxy (such as 2-nitrophenoxy, 4-nitrophenoxy, pentafluorophenoxy, 1-benzotriazolylloxy); alkylthio (such as S-tert-butyl) and arylthio (such as S-phenyl). Preferably R^6 is a dialkylamino group, more preferably a diisopropylamino group.

10

The group represented by R^6 on the coupling reagent (IV) will be displaced by a nucleophilic hydroxy group in M^2 -OH (such as an oligonucleotide hydroxy residue) to form the molecule represented by the formula (II). Preferably, the nucleophilic group is the 5'-OH terminus of the oligonucleotide residue.

15

The coupling reagents (III) and (IV) may be prepared by procedures such as those described in Reaction Schemes 2 and 3. It will be appreciated that coupling reagents of general formulae (III) and (IV) may be prepared by modification of the procedures shown in Reaction Schemes 1 and 2 in accordance with conventional synthetic organic chemistry.

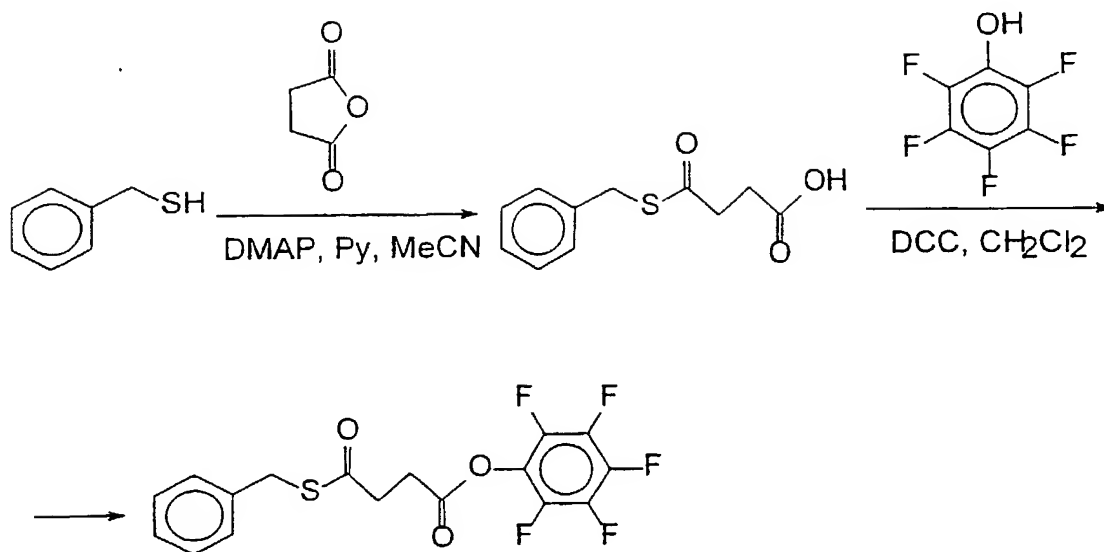
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In one aspect of the present invention, the compound of formula (IV) may be used to produce a compound of formula (II) by reaction with a second molecule M^2 -OH. The compound of formula (II) is then coupled with a compound of formula (I).

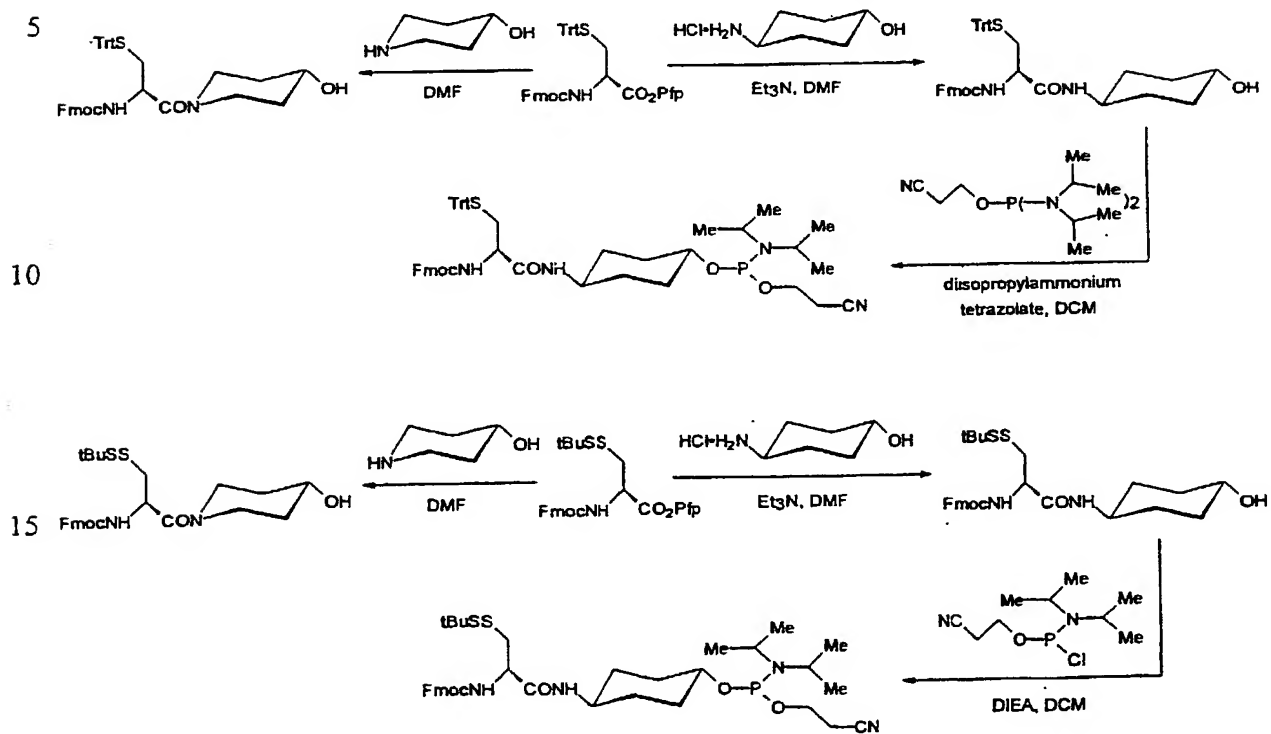
25 Alternatively, the compound of formula (IV) may be used for modifying molecules of type M^2 -OH, which are then coupled with a molecule of type M^1 -NH₂ that has been modified by a known route, for example a maleimido group as reported by Eritja *et al* (Tetrahedron, 47 (1991) 4113) and by Soukchareun *et al* (Bioconj. Chem. 9 (1998) 466).

30

According to a further aspect of the present invention, there is provided a kit comprising coupling reagent (III) and/or (IV). The kit may be used for modifying molecules such as M^1-NH_2 and M^2-OH , which can then be coupled by the native ligation reaction described herein. The kit may further comprise other components which may be used in the coupling
5 reaction. These include one or more components selected from thiophenol, TCEP, urea, guanidinium chloride and sodium phosphate buffer. The kit may also comprise instructions for carrying out the native ligation coupling reaction and/or instructions for modifying molecules such as M^1-NH_2 and M^2-OH .

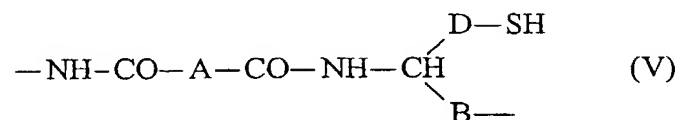


Reaction Scheme 2: Synthesis of coupling reagent (III)



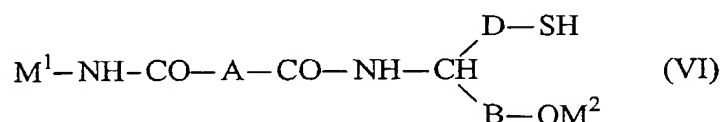
Reaction Scheme 3: Syntheses of coupling reagent (IV)

According to the present invention the coupling reaction provides a chemical compound comprising a structural unit of formula (V)



5 wherein A, B and D are as defined above.

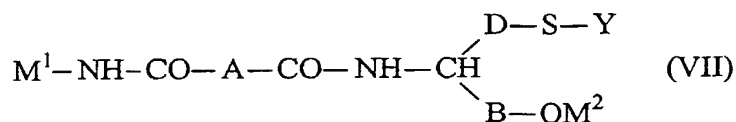
More particularly, there is provided a chemical compound of the formula (VI)



10 wherein M¹, M², A, B and D are as defined above.

Where M¹ and M² are peptide and oligonucleotide residues respectively, such compounds may have therapeutic utility. For example, the peptide residue may facilitate cellular uptake or intracellular activity of a therapeutic oligonucleotide. The peptide may act as a
 15 signal molecule for identification of the location of the oligonucleotide within a cell or for the delivery of the oligonucleotide to a specific cellular location or may enhance the targeting ability of the oligonucleotide towards cellular RNA or DNA in an antisense or triplex therapeutic or diagnostic application.

20 The thiol group of the compound of formula (VI) is particularly suited to further derivatisation or functionalisation and can, for example, provide a site for attachment of a labelling, reporter or effector group. Accordingly, the invention further provides a chemical compound of the formula (VII)



25

wherein M^1 , M^2 , A, B and D are as defined above, and Y is a labelling, reporter or effector group.

5 The invention will now be described in detail with reference to the following examples. It will be appreciated that this invention is described by way of example only and modification of detail may be made without departing from the scope of the invention.

10 **Example 1: Preparation of Coupling Reagent (III)**

1) S-Benzyl thiosuccinic acid. Benzyl mercaptane (22 mmol, 2,595 ml) was added under nitrogen to stirred solution of succinic anhydride (20 mmol, 2,0014 g) and 4-dimethylaminopyridine (1 mmol, 122.2 mg) in 25 ml of anhydrous acetonitrile – pyridine
15 (9:1 v/v). Stirring was continued at room temperature for 3 h, evaporated to near dryness, redissolved in sodium bicarbonate solution, pH 8.5, and extracted twice with diethyl ether. Water phase was then cooled at ice bath, acidified with 5N hydrochloric acid to pH 2, white precipitate filtered, washed with ice-cold water and dried in vacuum desiccator over phosphorus pentoxide overnight. Yield of white powder 3.5985 g (80%). $^1\text{H-NMR}$ (CDCl_3 ,
20 δ , ppm): 7.29-7.26 (m, 5H, Ph), 4.16 (s, 2H, CH_2S), 2.94-2.90 (t, 2H, CH_2COS), 2.77-2.72 (t, 2H, $\text{CH}_2\text{CO}_2\text{H}$).

2) Pentafluorophenyl S-benzyl thiosuccinate. Solution of dicyclohexylcarbodiimide (11 mmol, 2.2696 g) in 15 ml of dichloromethane was added dropwise to stirred and cooled
25 (ice bath) solution of S-benzyl thiosuccinic acid (2.243 g, 10 mmol) and pentafluorophenol (11.5 mmol, 2.117 g) in 25 ml of dichloromethane. Reaction mixture was stirred for 0.5 h on ice bath, then allowed to warm slowly to room temperature, stirred for 4 h and left overnight in a fridge. Dicyclohexylurea precipitate was filtered (2.148 g, 96% yield), solution concentrated in vacuo, redissolved in minimal volume of ethylacetate, filtered
30 again, and hexane was added. After standing overnight in a freezer, crystals were filtered, washed with cold ethylacetate - hexane (1:9 v/v) and dried in vacuo overnight. Yield of

white needles 3.4376 g (88%). After evaporating of mother liquor and further treatment with hexane additional 0.183 g of title compound could be obtained. Total yield of two crops 3.6206 g (92%). ¹H-NMR (CDCl₃, Δ , ppm): 7.30-7.27 (m, 5H, Ph), 4.18 (s, 2H, CH₂S), 3.05 (s, 4H, CH₂CH₂).

5

Example 2: Preparation of Coupling Reagent (IV)

1). N-Fmoc-S-trityl-L-cysteine 4-hydroxypiperidide. 4-Hydroxypiperidine (2.3 mmol, 232.6 mg) was added with stirring to the solution of N-Fmoc-S-trityl-L-cysteine pentafluorophenyl ester (2 mmol, 1.504 g) in 25 ml of anhydrous acetonitrile, followed by triethylamine (0.5 mmol, 0.07 ml). Reaction mixture was stirred at room temperature for 4 h, until TLC revealed the reaction completed. The mixture was then evaporated to dryness, redissolved in ethylacetate and washed successively with ice-cold 5% wt. citric acid, water, sodium bicarbonate and brine, dried over sodium sulfate, and evaporated to a light brown
15 foam. The residue was chromatographed on silica gel column eluted by 15-30% of acetonitrile in chloroform, appropriate fractions were pooled and evaporated to give 1.3126 g (98%) of the title product as a white foam.

2). N-Fmoc-S-trityl-L-cysteine 4-hydroxy-trans-cyclohexylamide. To the slurry of *trans*-4-aminocyclohexanol hydrochloride (2.1 mmol, 318.4 mg) and N-Fmoc-S-trityl-L-cysteine pentafluorophenyl ester (2 mmol, 1.504 g) in 25 ml of anhydrous DMF triethylamine was added (2.2 mmol, 0.307 ml), and resulting solution was stirred at room temperature for 3 h, until TLC revealed reaction completed. Reaction mixture was then evaporated to dryness, redissolved in ethylacetate and washed successively with ice-cold 5% wt. citric acid, water, sodium bicarbonate and brine, dried over sodium sulfate, and evaporated to a light brown
25 foam. The residue was chromatographed on silica gel column eluted by 15-5% hexane in ethylacetate – 0.5% triethylamine, appropriate fractions were pooled and evaporated to give 1.3045 g (95%) of the title product as a white foam.

3). N-Fmoc-S-*tert*-butylsulfenyl-L-cysteine 4-hydroxy-trans-cyclohexylamide. To the slurry of *trans*-4-aminocyclohexanol hydrochloride (2 mmol, 303.3 mg) and N-Fmoc-S-*tert*-butylsulfenyl-L-cysteine pentafluorophenyl ester (2 mmol, 1.195 g) and 1-hydroxybenzotriazole (2 mmol, 270.3 mg) in 20 ml of anhydrous DMF, triethylamine was
30

added (3.1 mmol, 0.446 ml), and resulting solution was stirred at room temperature for 3 h, until TLC revealed reaction completed. Reaction mixture was then evaporated to dryness, white residue transferred to sintered glass filter, washed successively with small amount of DMF, ethanol and diethyl ether, and dried in vacuo. Yield of white powder 0.868 g (82%).

- 5 4). N-Fmoc-S-*tert*-butylsulfenyl-L-cysteine 4-hydroxypiperidide. 4-Hydroxypiperidine (2.5 mmol, 253.1 mg) was added with stirring to the solution of N-Fmoc-S-*tert*-butylsulfenyl-L-cysteine pentafluorophenyl ester (2 mmol, 1.195 g) in 20 ml of anhydrous DMF. Reaction mixture was stirred for 3 h at room temperature until TLC revealed completed reaction. The mixture was then evaporated to dryness, redissolved in
10 ethylacetate and washed successively with 5% wt. citric acid, water, sodium bicarbonate and brine, dried over sodium sulfate, and evaporated to a light yellow foam. The residue was chromatographed on silica gel column eluted by 10-0% hexane in ethylacetate, appropriate fractions were pooled and evaporated to give 0.9831 g (95%) of the title product as a white foam.
- 15 5). 4-*trans*-N-Fmoc-S-trityl-L-cysteinylamidocyclohexyl 2-cyanoethyl N,N-diisopropyl phosphoramidite. To a solution of N-Fmoc-S-trityl-L-cysteine 4-hydroxy-*trans*-cyclohexylamide (0.3444 mmol, 0.2352 g) in 10 ml of anhydrous dichloromethane containing 75 mg (1.5 eq) of diisopropylammonium tetrazolate, 2-cyanoethoxy-N,N,N',N'-tetraisopropyl phosphordiamidite (1.15 eq, 0.126 ml) was added, and the
20 mixture was stirred for 6 h at room temperature, until TLC revealed complete conversion. Dichloromethane was then evaporated, residue taken up in ethylacetate, washed with saturated sodium bicarbonate and brine, dried over anhydrous sodium sulfate and evaporated to a small volume. The rest was chromatographed on silica gel column eluted with 25-10% hexane in ethylacetate – 3% triethylamine, appropriate fractions pooled and
25 evaporated to dryness. Yield of a title product 1.3045 g (95%).
- 6). 4-N-Fmoc-S-trityl-L-cysteinylpiperidyl 2-cyanoethyl N,N-diisopropyl phosphoramidite. To a chilled (ice bath) solution of N-Fmoc-S-trityl-L-cysteine 4-hydroxypiperidide (0.668 g, 1 mmol) in 10 ml of anhydrous dichloromethane containing 3
mmol (0.514 ml) of diisopropylethylamine, 2-cyanoethoxy-N,N-
30 diisopropylaminochlorophosphine (1.3 mmol, 0.29 ml) was added dropwise via syringe under nitrogen. After 1 h of stirring cold, the mixture was allowed to warm up gradually,

and stirring was continued for 2 h at room temperature. The mixture was then quenched with 0.1 ml of methanol, evaporated to dryness, residue taken up in ethylacetate, washed with saturated sodium bicarbonate and brine, dried over anhydrous sodium sulfate and evaporated to a small volume. The rest was chromatographed on silica gel column eluted with 30-15% hexane in dichloromethane – 3% triethylamine, appropriate fractions pooled and evaporated to dryness. Yield of a title product 0.695 g (80%).

7). 4-trans-N-Fmoc-S-tert-butylsulfenyl-L-cysteinyamidocyclohexyl 2-cyanoethyl N,N-diisopropyl phosphoramidite. To a chilled (ice bath) solution of 4-trans-N-Fmoc-S-tert-butylsulfenyl-L-cysteinyamidocyclohexanol (0.834 g, 1.577 mmol) in 15 ml of anhydrous dichloromethane containing 3 eq (0.785 ml) of diisopropylethylamine, 2-cyanoethoxy-N,N-diisopropylamino chlorophosphine (1.5 eq, 0.529 ml) was added dropwise via syringe under nitrogen. After 1 h of stirring cold, the mixture was allowed to warm up gradually, and stirring was continued for 2 h at room temperature. The mixture was then quenched with 0.1 ml of methanol, evaporated to dryness, residue taken up in ethylacetate, washed with saturated sodium bicarbonate and brine, dried over anhydrous sodium sulfate and evaporated to a small volume. The rest was chromatographed on silica gel column eluted with 25-10% hexane in ethylacetate – 2.5% triethylamine, appropriate fractions pooled and evaporated to dryness. Yield of a title product 0.7567 g (66%).

8) 4-N- α -Fmoc-S-tert-butylsulfenyl-L-cysteinylpiperidyl 2-cyanoethyl N,N-diisopropyl phosphoramidite. To a solution of N- α -Fmoc-S-tert-butylsulfenyl-L-cysteine 4-hydroxypiperidide (1.536 mmol, 0.790 g) in 15 ml of anhydrous CH₂Cl₂ containing 0.329 g (1.920 mmol) of diisopropylammonium tetrazolide, 2-cyanoethoxy-N,N,N',N'-tetraisopropyl phosphordiamidite (1.690 mmol, 0.537 ml) was added under N₂ atmosphere, and the mixture was stirred for 3 h at room temperature, until TLC (B) revealed complete reaction. The solvent was then removed by evaporation, the residue was taken up in EtOAc, washed with saturated NaHCO₃ solution (x2) and brine, dried over anhydrous Na₂SO₄, filtered through a cotton plug, evaporated to a white foam, re-evaporated several times with CH₂Cl₂, dried *in vacuo* and dissolved in small volume of 10% EtOAc in hexane. The solution was chromatographed on a silica gel column eluted with 10-40% EtOAc in hexane containing 2% triethylamine, appropriate fractions were pooled, evaporated to dryness and rinsed with hexane. The remaining waxy solid was dissolved in

dry CH_2Cl_2 , and re-evaporated twice with dry CH_2Cl_2 to give a white foam. Yield of title product 0.455 g (42%). TLC (B): R_f 0.78. ^1H NMR (CD_3CN): δ 1.15-1.19 (m, 12), 1.33 (s, 9), 1.61 (bm, 2), 1.82 (bm, 2), 1.93-1.97 (quintet, 1), 2.16 (s, MeCN), 2.61-2.67 (t, 2), 2.81-2.88 (m, 1), 3.04-3.10 (m, 1), 3.44 (bm, 2), 3.57-3.80 (bm, 6), 4.09 (m, 1), 4.22-4.26 (t, 1), 4.35-4.37 (d, 2), 4.86-4.88 (quadruplet, 1), 6.12-6.17 (t, 1), 7.32-7.37 (t, 2), 7.40-7.45 (t, 2), 7.66-7.68 (d, 2), 7.83-7.85 (d, 2). ^{31}P NMR (CD_3CN): δ 147.10 (61%), 147.03 (19%), 146.94 (20%) (mixture of rotamers). MALDI-TOF MS: (M+H) 715.7 (715.9 calc.), (M+Na) 737.0 (737.9 calc.), (M+K) 753.4 (754.0 calc.).

10 Example 3: Preparation of Modified Peptide (I)

All peptides were synthesized on the PioneerTM peptide synthesizer (PE Biosystems) on 0.1 mmol scale, by HATU/DIEA mediated in situ activation protocol supplied by manufacturer. N- α -Fmoc amino acids had standard side chain protection, respectively: 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl on arginine, trityl on asparagine and glutamine, t-butyl on aspartic and glutamic acids, serine, threonine and tyrosine, t-butoxycarbonyl on lysine and tryptophan, and trifluoroacetyl on lysine, were used in 4-fold excess, with no double couplings, and all were of commercial origin. Syntheses were made on either PAL-PEG-PS support (PE Biosystems) or Rink amide NovaGelTM (CN Bioscience). After completion of the desired sequence and N-terminal Fmoc deprotection, the support (0.1 mmol) was transferred to a vial, and then coupling of pentafluorophenyl S-benzylthiosuccinate was performed manually, using 4.5 eq (0.176 g) of the compound and 1 eq (14 mg) of 1-hydroxy-7-azabenzotriazole (HOAt) as a catalyst, in 2 ml of a freshly distilled DMF for 4 h at room temperature. The resin was then washed with 5x4 ml of DMF, 5x3 ml of methanol, and 5x2 ml of diethyl ether, and dried in vacuo. The N-modified peptide was cleaved from the resin and deprotected, using 6 ml of trifluoroacetic acid – benzyl mercaptan – phenol – water (90:5:2.5:2.5 v/v/v/v) cocktail, for 2-6 h at room temperature, depending on arginine content. The resin was filtered, washed with 5x4 ml of TFA, the volume was reduced to cal. 1-2 ml by purging nitrogen, and 40 ml of cold diethyl ether was then added to precipitate the peptide. The slurry was centrifugated, ether decanted, and procedure was repeated three times to remove scavengers. The peptide

pellet was then dried in vacuo, dissolved in 0.1% aqueous TFA/acetonitrile mixture, and subjected to RP HPLC purification on a C₈ column by a gradient of an acetonitrile in 0.1% aqueous TFA. The appropriate fractions were lyophilised and analysed by MALDI-TOF MS, using α -cyano-4-hydroxy cinnamic acid as a matrix. Typical examples are given in

5 **Table 1.**

Table 1. Automated solid phase synthesis of peptide N-terminal S-benzyl thioesters.

Sequence	MALDI-TOF MS	Purity of crude product, % (HPLC)
BnSCO-RQIPK ^{11a} TWFPNRRK ^{11a} PFK ^{11a} K ^{11a} -CONH ₂	2714.59	72.0
BnSCO-GRK ^{11a} K ^{11a} RRQRRR-CONH ₂	1791.63	76.5
BnSCO-PTSQSRGDPTGPKE-CONH ₂	1660.25	88.7
BnSCO-DRVIEVVQGAYRAIRNIPRRIRQG-CONH ₂	3040.62	28.6
BnSCO-QAKKKKLDK-CONH ₂	1289.92	90.3
BnSCO-ALPPLERLTL-CONH ₂	1325.70	74.7
BnSCO-GALFLGFLGAAGSTMGAWSQPKSKRKV-CONH ₂	2969.05	48.3
BnSCO-Sar-Leu-Gly-Ile-Gly-CONH ₂	657.91	92.2
BnSCO-PQIK ^{11a} TWFPNRRK ^{11a} PFK ^{11a} K ^{11a} -CONH ₂	2715.02	72.0

Example 4: Preparation of Modified Oligonucleotide (II)

Oligonucleotides were synthesized on ABI 380B automated DNA/RNA synthesizer (PE Biosystems) on 1 μ mol scale. Standard 3'-nucleoside succinate LCAA-CPG 500A supports were used. After completion of the assembly of the desired sequence and 5'-terminal 4,4'-dimethoxytrityl (Dmt) group cleavage by 2% dichloroacetic acid solution in dichloromethane (v/v), support-bound oligonucleotide was treated on machine with 0.15M solution of modified phosphoramidite reagent (t-butylsulfenyl or trityl S-protected) in anhydrous acetonitrile mixed with 0.5M solution of 1H-tetrazole in anhydrous acetonitrile, for 10 min at room temperature. The support was then washed with standard oxidizing solution (0.1M iodine in aqueous pyridine – tetrahydrofuran), washed with acetonitrile and dried. Fmoc amino protecting group can be selectively removed from support-bound oligonucleotide by treating the support with 20% piperidine solution in dimethylformamide (v/v) for 15 min at room temperature. S-Trityl protecting group can be selectively removed on solid support with 0.05M aqueous solution of silver nitrate for 15 min, followed by 0.05M dithiothreitol (DTT) for 5 min (Mag *et al*, Nucl. Acids Res., 19 (1991) 1437). Alternatively, S-Trt protection can be removed in solution with silver nitrate as well (Connolly and Rider, Nucl. Acids Res., 13 (1985) 4485). S-t-Butylsulfenyl group is also sufficiently stable to concentrated aqueous ammonia deprotection at 55°C routinely employed in oligonucleotide synthesis to allow isolation of S-t-butylsulfenyl-protected oligonucleotides, which may be subsequently deprotected by treating with 0.1M aqueous solution of tris-(2-carboxyethyl)phosphine, pH 7.0, at room temperature overnight. Both S-trityl and S-t-butylsulfenyl provide convenient hydrophobic handles for reverse phase purification of synthesized oligonucleotides. Routinely, S-protected cysteine-modified oligonucleotides were isolated after RP HPLC on μ Bondapak C₁₈ column eluted by a gradient of an acetonitrile in 0.1M aqueous ammonium or triethylammonium acetate solution, pH 7.0, desalted on Sephadex G-10 or G-25 column, lyophilised and analysed by MALDI-TOF MS using 2,6-dihydroxyacetophenone – ammonium citrate as matrix. Typical examples are given in Table 2.

Table 2. Automated solid phase synthesis of 5'-cysteinyl oligonucleotides.

Sequence	MALDI-TOF MS	Purity of crude product, % (HPLC)
Cys(SBu ¹)-TTT TT	1825.13	87.1
Cys(Trt)-TTT TT	1983.16	76.6
Cys-TTT TT	1738.24	82.2
Cys(SBu ¹)-CTC CCA GGC TCA AAT	4874.42	82.6
Cys(SBu ¹)-GCT CCC AGG CTC AAA	4888.15	89.7
Cys(Trt)-CTC CCA GGC TCA AAT	5017.05	91.2
Cys(Trt)-GCT CCC AGG CTC AAA	5043.74	89.2
Cys(Trt)-AGC TCC CAG GCT CAA	5043.88	90.5
Cys(SBu ¹)-AGC TCC CAG GCT CAA	4887.98	86.5
Cys-GCT CCC AGG CTC AAA	4803.15	100

Example 5: Coupling Reactions (Solution phase)

5

The solution phase peptide-oligonucleotide coupling reactions were performed using four different experimental procedures (Methods A, B, C and D). Typical examples are given in **Table 3**.

Table 3. Synthesis of Peptide-N-to-5'-oligonucleotide conjugates

10

Oligonucleotide sequence	Peptide Sequence	Method	MALDI-TOF MS	Yield, %
TTT TT	PTSQSRGDPTGPPKE	A	3276.72	75
GCT CCC AGG CTC AAA	PTSQSRGDPTGPPKE	B	6340.17	65
GCT CCC AGG CTC AAA	Sar-Leu-Gly-Ile-Gly	C	5349.05	26
GCT CCC AGG CTC AAA	ALPPLERLTL	C	6043.43	22
AGC TCC CAG GCT CAA	Sar-Leu-Gly-Ile-Gly	D	5348.45	43
AGC TCC CAG GCT CAA	ALPPLERLTL	D	6043.88	51

Example 5a (Method A). 0.9 μ mol of crude oligodeoxynucleotide TTT TT, 5'-modified with 4-(S-*tert*-butylsulfenyl)cysteinylamido-*trans*-cyclohexyl phosphate, were dissolved in 1 ml of 25% v/v N,N-dimethylformamide – 0.1M sodium phosphate buffer, pH 7.5, containing 7M urea and 0.1M *tris*-(2-carboxyethyl)phosphine, and 4.5 μ mol of lyophilised

15

peptide PTSQSRGDPTGPKE amide, N-terminally modified with S-benzyl thiosuccinyl moiety, were added followed by 4% v/v thiophenol. The mixture was incubated at ambient temperature for 24 h, and then analysed by RP-HPLC (μ Bondapak C₁₈ analytical column, detection at 218 and 254 nm, flow rate 1 ml/min, buffer A: 0.1M ammonium acetate, pH 7.0, buffer B: acetonitrile, gradient: 5 min 2% B, 20 min 40% B, 25 min 100% B, retention time: starting *S-tert*-butyl oligonucleotide 16.608 min, reduced oligonucleotide 14.575 min, conjugate 15.258 min). After isolation by preparative HPLC, conjugate was assessed by MALDI-TOF MS: calculated 3277.59, observed 3376.72. Yield: 75% calculated on starting oligonucleotide.

- 10 **Example 5b (Method B).** 0.6 μ mol of crude oligodeoxynucleotide GCTCCCAGGCTCAAA, 5'-modified with 4-(*S-tert*-butylsulfenyl)cysteinylamido-*trans*-cyclohexyl phosphate, were dissolved in 1 ml of 25% v/v N,N-dimethylformamide – 0.1M sodium phosphate buffer, pH 7.5, containing 0.1M *tris*-(2-carboxy ethyl)phosphine, and 3 μ mol of lyophilised peptide PTSQSRGDPTGPKE amide, N-terminally modified with S-benzyl thiosuccinyl moiety, were added followed by 4% v/v thiophenol. The mixture was incubated at ambient temperature for 24 h, and then analysed by RP-HPLC (μ Bondapak C₁₈ analytical column, detection at 218 and 254 nm, flow rate 1 ml/min, buffer A: 0.1M triethylammonium acetate, pH 7.0, buffer B: acetonitrile, gradient: 5 min 2% B, 20 min 40% B, 25 min 100% B, retention time: starting *S-tert*-butyl oligonucleotide 16.875 min, reduced oligonucleotide 16.083 min, disulfide oligonucleotide 24.750 min, conjugate 15.541 min, disulfide conjugate 26.933 min). After isolation by preparative HPLC, conjugate was assessed by MALDI-TOF MS: calculated 6340.51, observed 6340.17. Yield: 65% calculated on starting oligonucleotide.

- 25 **Example 5c (Method C)** The coupling reactions were performed under the following conditions: 0.05 mM modified oligonucleotide, 10 equiv. modified peptide, 0.1 M TCEP, titrated to pH 6.5 by addition of 20% sodium hydroxide solution, 2% PhSH (v/v), 25% acetonitrile, 48 h, room temperature.

- Example 5d (Method D)** The coupling reactions were performed under the following conditions: 0.01 mM modified oligonucleotide, pretreated with 0.2 M TCEP, pH 6.5, for 30 3 h at room temperature, then 10 equiv. modified peptide in an equal volume of 50%

aqueous DMF was added together with PhSH (2% v/v final) and kept at room temperature for 48 h.

Example 6: Coupling reaction (solid phase)

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Solid phase oligonucleotide synthesis column with 1 μ mol of crude oligodeoxynucleotide CTCCCAGGCTCAAAT, 5'-modified with 4-(N-Fmoc-S-*tert*-butylsulfenyl)cysteinyllamido-*trans*-cyclohexyl 2-cyanoethyl phosphate, still attached to the support, was treated in syringe first with 20% piperidine in N,N-dimethylformamide (v/v) for 15 min, washed with 5 ml DMF, then with 1 ml of 0.5M solution of dithiothreitol in N,N-dimethylformamide – water (1:1 v/v) for 2 h, and washed with 10 ml DMF – water (1:1 v/v). Then 1 ml of solution of 5 μ mol of lyophilised peptide GRK^{Tfa}K^{Tfa}RRQRRR amide (Tfa – trifluoroacetyl), N-terminally modified with S-benzyl thiosuccinyl moiety, in 25% DMF – 0.1M sodium phosphate buffer, pH 7.5, containing 4% v/v thiophenol, was added via syringe. The column was incubated at ambient temperature for 24 h, and then washed with 10 ml of 25% acetonitrile – water (v/v). Then 1 ml of 0.5M iodoacetamide solution in 40% DMF – 0.1M sodium phosphate buffer, pH 7.5, was added via syringe, and the column incubated at ambient temperature for further 24 h. After washing with 10 ml of DMF – water (1:1 v/v) support was dried, transferred to screw-capped vial and treated with 25% aqueous ammonia solution at 55°C for 16 h. The glass beads were decanted, washed with 0.5 ml of 25% aqueous ammonia and 0.5 ml of water, the volume of supernatant was reduced to 250 μ l, and the mixture was then analysed by RP-HPLC (μ Bondapak C₁₈ analytical column, detection at 218 and 254 nm, flow rate 1 ml/min, buffer A: 0.1M ammonium acetate, pH 7.0, buffer B: acetonitrile, gradient: 5 min 2% B, 20 min 40% B, 25 min 100% B, retention time: starting S-*tert*-butyl oligonucleotide 15.025 min, reduced oligonucleotide 13.338 min, conjugate 14.016 min). Yield: cal. 45% by HPLC.

Example 7: Alternative Coupling Reaction

30 A 3'-fluoresceinyl 15-mer oligodeoxyribonucleotide dCTCCCAGGCTCAAAT was synthesised on a 1 μ mol scale using a commercially available fluorescein-derivatised

controlled poor glass support. Then the cysteinylpiperidine phosphoramidite reagent (t-butylsulfenyl protected) was coupled and, after standard iodine oxidation, the terminal Fmoc group was removed by brief treatment with piperidine. Following standard aqueous ammonia deprotection and release into solution, the resultant 5'-S-tert-butylthiocysteine
5 3'-fluorescein oligonucleotide derivative showed by reversed phase HPLC a single main peak at 18.3 minutes elution time. After HPLC purification, the yield was 20.7 A₂₆₀ units. The product showed a single peak by HPLC and a single band by polyacrylamide gel electrophoresis. The MALDI-TOF mass spectra showed a mass of 5424 Da (calculated 5422). When this oligonucleotide was treated with 0.2 M TCEP solution (pH 6.5) for 30
10 min at room temperature, the free thiol was obtained quantitatively as observed by HPLC (elution time 16.7 min). The conjugation reaction of this 5'-cysteine-3'-fluorescein oligodeoxynucleotide with maleimide peptides in aqueous solution by methods similar to those previously reported (Soukchareun *et al*, Bioconj. Chemistry, 9, 1998, 466-475) showed that the oligonucleotide was in each case completely consumed within 16 h.

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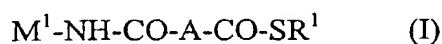
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CLAIMS

1. A method of linking a first molecule M^1-NH_2 with a second molecule M^2-OH comprising reaction of a compound of formula (I)

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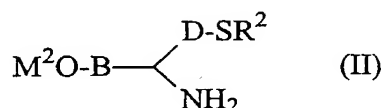
wherein M^1 is the residue of a molecule bearing an amino group

A is an alkylene or arylene group,

10

R^1 is alkyl or aryl,

with a compound of formula (II)



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M^2 is the residue of a molecule bearing a hydroxy group

wherein B is a linker

D is a C_{1-4} alkylene group or C_{3-12} arylene group

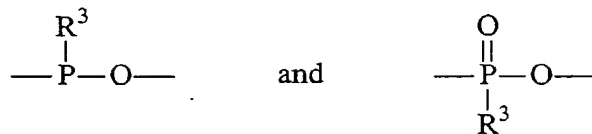
R^2 is hydrogen or a thiol protecting group.

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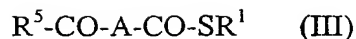
2. A chemical compound of formula (I) as defined in claim 1.
3. A chemical compound according to claim 2 wherein M^1 comprises a peptide residue.
- 25 4. A chemical compound according to claim 2 or 3 wherein A comprises a C_{1-4} alkylene group.
5. A chemical compound according to claim 4 wherein A comprises an ethylene ($-CH_2-CH_2-$) or n-propylene ($-CH_2-CH_2-CH_2-$) group.
- 30

6. A chemical compound according to any one of claims 2 to 5 wherein R^1 comprises a C_{1-18} alkyl or C_{3-10} aryl group.
- 5 7. A chemical compound according to claim 6 wherein R^1 is selected from the group consisting of t-butyl, substituted or unsubstituted benzyl, substituted or unsubstituted phenyl, 2-pyridyl, 4-pyridyl, cyanomethyl carboxamidomethyl, 2-carboxamidoethyl and trifluoroethyl.
- 10 8. A chemical compound of formula (II) as defined in claim 1.
9. A chemical compound according to claim 8 wherein M^2 comprises an oligonucleotide residue.
- 15 10. A chemical compound according to claim 8 or 9 wherein the linker B comprises a group of the formula
- X-J-
- 20 wherein J is an alkylene or arylene group
X is the residue of a functional group capable of reacting with a hydroxy group.
11. A chemical compound according to claim 10 wherein J is a C_{1-18} alkylene group or
- 25 C_{3-12} arylene group.
12. A chemical compound according to claim 11 wherein J comprises a moiety derived from trans-4-aminocyclohexanol or 4-hydroxypiperidine.
- 30 13. A chemical compound according to any one of claims 10 to 12 wherein X is a phosphate, thiophosphate, phosphonate or phosphite residue.

14. A chemical compound according to claim 13 wherein X comprises a group selected from



- 5 wherein R³ is selected from hydroxy, oxy anion and salts thereof, alkyl, alkoxy, aryloxy, thiol, thioxy anion and salts thereof, S-alkyl, S-aryl, N-azolyl, dialkyl amino groups.
15. A chemical compound according to claim 14 wherein R³ is a 2-cyanoethoxy group.
- 10 16. A chemical compound according to any one of claims 8 to 15 wherein D is a methylene or ethylene group.
17. A chemical compound according to any one of claims 8 to 16 wherein R² is
15 selected from hydrogen, alkyl, S-alkylsulfenyl, S-arylsulfenyl, alkylcarboxamidoalkyl, urethanyl and acyl groups.
18. A chemical compound according to claim 17 wherein R² is hydrogen tert-butyl
20 sulfenyl or trityl.
19. A chemical compound according to any one of claims 8 to 18 wherein the amino
group of the compound of formula II is protected.
20. A chemical compound according to claim 20 wherein the amino group is protected
25 with a protecting group R⁴ selected from urethanyl, alkyl, alkylsulfenyl, aryl sulfenyl and sulfonyl protecting groups.
21. A chemical compound of the formula (III)

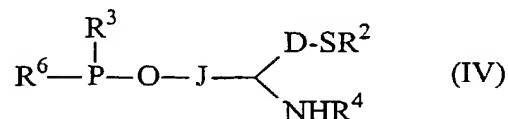


wherein A and R¹ are as defined in any preceding claim

R⁵ is selected from hydroxy, oxy anion and salts thereof, alkoxy, aryloxy N-succinimidyloxy, N-(norbornenedicarboximido)oxy, N-benzotriazolyloxy, N-(1,2-dihydro-1-oxo-2,3,4-benzotriazin-2-yl)oxy, halogen and N-azolyl groups; or together with the adjacent CO group forms an anhydride.

22. A chemical compound according to claim 21 wherein R⁵ is a pentafluorophenoxy group.

23. A chemical compound of the formula (IV)



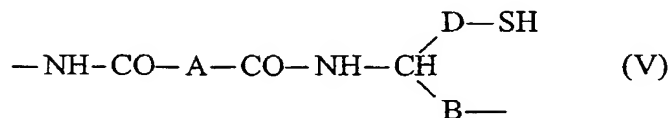
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wherein D, J, R², R³ and R⁴ are as defined in any preceding claim, R⁶ is selected from dialkyl amino, imino, halogen, N-azolyl, alkoxy, aryloxy, alkylthio, arylthioaryl groups.

24. A chemical compound according to claim 23 wherein R⁶ is a dialkyl amino group.

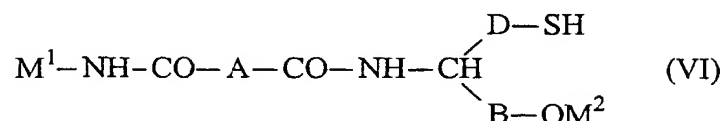
25. A chemical compound according to claim 24 wherein R⁶ is a di-isopropylamino group.

26. A chemical compound comprising a structural unit of formula (V)



wherein A, B and D are as defined in any preceding claim.

27. A chemical compound according to claim 26 of the formula (VI)

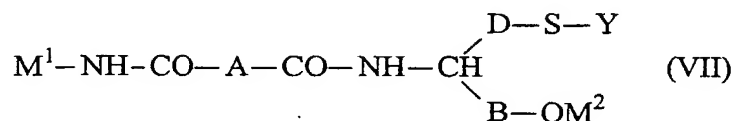


- 5 wherein M^1 , M^2 , A, B and D are as defined in any preceding claim.

28. A chemical compound according to any one of claims 2 to 27 wherein the compound is linked to a solid support.

- 10 29. A method according to claim 1 wherein M^1 , M^2 , A, B, D, R^1 and R^2 are as defined in any of claims 4 to 23.

30. A chemical compound of the formula (VII)



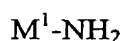
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- wherein M^1 , M^2 , A, B and D are as defined in any preceding claim
Y is labelling, reporter or effector group.

- 20 31. Use of a chemical compound according to any one of claims 2 to 28 in a process for linking a peptide and an oligonucleotide.

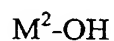
32. A chemical compound according to claim 27 or 30 for use in therapy.

- 25 33. A method of producing a compound of formula (I) comprising reaction of a compound of the formula



with a chemical compound according to claim 21 or 22 wherein M^1 , A, R^1 and R^5 are as defined in any preceding claim.

34. A method of producing a compound of formula II comprising reaction of a compound of the formula



- with a chemical compound according to any one of claims 23 to 25 wherein M^2 , B, D, R^2 , R^3 , R^4 and R^6 are as defined in any preceding claim.

35. A kit comprising a chemical compound according to claim 21 or 22 and/or a chemical compound according to any one of claims 23 to 25.

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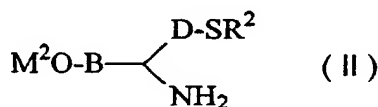
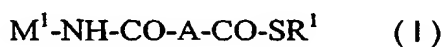
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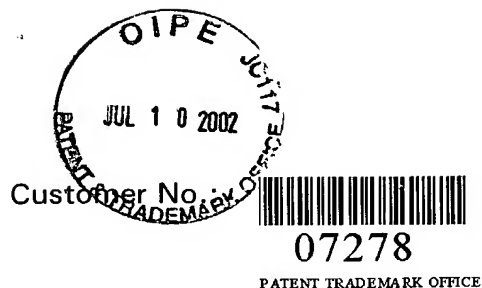
For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: **METHOD FOR COUPLING MOLECULES**



(57) Abstract: A method of linking a first molecule M^1-NH_2 with a second molecule M^2-OH comprising reaction of a compound of the formula (I): $M^1-NH-CO-A-CO-SR^1$ wherein M^1 is the residue of a molecule bearing an amino group, A is an alkylene or arylene group, R^1 is alkyl or aryl, with a compound of formula (II), M^2 is the residue of a molecule bearing a hydroxy group wherein B is a linker, D is C_{1-4} alkylene group or C_{3-12} arylene group, R^2 is hydrogen or a thiol protecting group. In addition, this invention relates to conjugate products of the coupling reaction, reagents for modifying M^1-NH_2 and M^2-OH , and kits comprising these reagents.

WO 01/15737 A2



Docket No.: 2224/OK248

**DECLARATION
AND POWER OF ATTORNEY
Original Application**

As a below named inventor, I declare that the information given herein is true, that I believe that I am the original, first and sole inventor if only one name is listed at 1 below, or a joint inventor if plural inventors are named below, of the invention entitled:

METHOD FOR COUPLING MOLECULES

which is described and claimed in:

☐ the attached specification or ☒ the specification in application Serial No. 10/070,222, filed February 27, 2002

(for declaration not accompanying appl.)

that I do not know and do not believe that the same was ever known or used in the United States of America before my or our invention thereof or patented or described in any printed publication in any country before my or our invention thereof, or more than one year prior to this application, or in public use or on sale in the United States of America more than one year prior to this application, that the invention has not been patented or made the subject of an inventor's certificate issued before the date of this application in any country foreign to the United States of America on an application filed by me or my legal representatives or assigns more than twelve months prior to this application, that I acknowledge my duty to disclose information of which I am aware which is material to patentability in accordance with 37 CFR §1.56. I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above. I hereby claim the priority benefits under 35 U.S.C. §119 of any application(s) for patent or inventor's certificate listed below. All foreign applications for patent or inventor's certificate on this invention filed by me or my legal representatives or assigns prior to the application(s) of which priority is claimed are also identified below.

PRIOR APPLICATION(S), IF ANY, OF WHICH PRIORITY IS CLAIMED

<u>COUNTRY</u>	<u>APPLICATION NO.</u>	<u>DATE OF FILING</u>
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**ALL FOREIGN APPLICATIONS, IF ANY, FILED PRIOR
TO THE APPLICATION(S) OF WHICH PRIORITY IS CLAIMED**

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Great Britain	0012083.2	May 18, 2000

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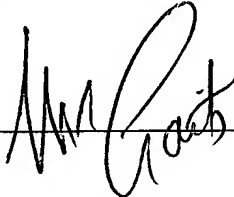
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I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

SIGNATURE OF INVENTOR 1:



DATED:

1 / MAY / 2002

SIGNATURE OF INVENTOR 2:



DATED:

1 / MAY / 2002